# Structure and Dynamics of Pure Hybridogenetic Water Frog Populations of *Rana esculenta* in Southern Sweden

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**Dissertation**

**zur**

**Erlangung der naturwissenschaftlichen Doktorwürde (Dr.sc.nat.)**

**vorgelegt der**

**Mathematisch-naturwissenschaftlichen Fakultät**

**der**

**Universität Zürich**

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**Zürich 2007**

**„Jag är en skönhet och han en groda…**

**Vad mer behövs?“**

Gittan, i „Miljardärsgrodan“

Kalle Ankas Pocket Dubbel 298, 2004

Die vorliegende Arbeit wurde von der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich am 1. Oktober 2007 als Dissertation angenommen.

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# General introduction

## *Hybridization and speciation*

Interspecific hybridization has been considered a main driving force in the speciation of plants for a long time (Anderson and Stebbins 1954, Grant 1971). But the role of hybridization in animal evolution was long dismissed as insignificant and considered as an evolutionary dead-end (Mayr 1963). Due to their frequently intermediate morphological, physiological and behavioural characteristics (Arnold 1997, Vences et al. 2003, Mavárez et al. 2006), hybrids are often at a selective disadvantage, at least in the parental species' habitat. Besides the exogenous selection against hybrids, there is also negative endogenous selection (Bronson et al. 2003, Peterson et al. 2005): The disruption of advantageous gene combinations, negative epistasis and chromosomal mispairing due to the combination of two "alien genomes" may result in offspring that are inviable or infertile and, hence, render hybrids less fit than their parents. But occasionally, hybrids are even more fit than their parental species, even in the parental habitat (see reviews in Barton 2001, Burke and Arnold 2001, Seehausen 2004). In recent years, it was discovered that also many animal taxa underwent hybridization events in their evolutionary past (reviews by Arnold 1997, Mallet 2005), including primates (Arnold and Meyer 2006).

To explain the evolutionary success and longevity of some hybrid taxa (Hedges et al. 1992, Bullini 1994, Schartl et al. 1995, Alves et al. 2001, Janko et al. 2003), several models have been postulated. The tension zone model assumes that hybrids are selected against, but their presence is maintained by constant new formation in an area of overlap of the parental species (Barton and Hewitt 1985). According to the bounded hybrid superiority model (Moore 1977), hybrids are inferior and less fit in the parental habitat, but more fit than their parental phenotypes within the hybrid zone, which is usually a narrow, ecotonal zone. As a possible result, the habitat is partitioned among the different taxa, with the hybrid using an intermediate niche. Finally, the mosaic model (Harrison and Rand 1989) assumes no smooth transition, but patchy mosaics of different habitats in hybrid zones. Different habitat preferences of the hybrid and its

parental species therefore lead to a patchy distribution of genotypes in the contact zone. While the tension zone model may in fact lead to a reinforced species boundary between the parental species, the other models actually allow for an evolutionary success of the hybrid taxon.

The occurrence of hybridization usually also has an impact on the parental species and their population dynamics and stability. Depending on the hybrids' fitness and the overlap of their niches, backcrossing with its parental species may occur. The resulting introgression of new alleles into a well adapted genome can either have negative consequences for the species in this habitat and lead to a population decrease, or it broadens the species niche and leads to an increased population fitness (Dowling and Secor 1997, Barton 2001, Turelli et al. 2001, Mallet 2005, Arnold and Meyer 2006). Hybridization between species is of special importance when one of the hybridizing species is invasive. Such genetic interactions can lead to increased invasiveness and alter future patterns of hybridization (Lambrinos 2004). For plants as well as for animals, there are examples which indicate that an initial hybridization event can be followed by an impressive adaptive radiation and lead to the establishment of many new species (Ellstrand and Schierenbeck 2000, Seehausen 2004).

For such a successful establishment of hybrid populations, two main problems must be solved. First, the inherent spatial proximity to the parental species requires that the hybrid becomes reproductively isolated to prevent that the hybrid genomes are constantly swamped by gene flow from the parental populations. In some cases, intermediate phenotypic traits are already an effective reproductive barrier, such as in Heliconius butterflies (Mavárez et al. 2006). Spatial habitat or niche portioning is another possibility for isolation. A further requirement for independent reproduction of hybrids is that they overcome possible meiotic chromosome pairing difficulties. Both problems, reproductive isolation as well as chromosome pairing difficulties, can be overcome by polyploidy or by parthenogenesis (Kearney 2005). Parthenogenesis may potentially also fix any hybrid vigour effects (heterosis) of the F1-generation.

## *Hybrids and hybridogens in European water frogs*

When Carl von Linné 1758 described the edible frog *Rana esculenta* in Sweden, he considered it to be an independent biological species. At that time, *R. esculenta* was the first water frog taxon to be described, followed by *Rana ridibunda* Pallas in 1771, *Rana hispanica* Bonaparte in 1839 and *Rana lessonae* Camerano in 1882. There was always some discussion about the relationships between the described water frogs because they are morphologically and behaviorally very similar to each other (Kauri 1959, Mertens and Wermuth 1960). However, it wasn't until the 20$^{th}$ century that it was shown that *R. esculenta* is not a true species, but rather of hybrid origin. Berger (1967) performed first crossing experiments between the three water frog taxa *R. esculenta*, *R. ridibunda* and *R. lessonae* and found that *R. esculenta* is a hybrid between the two true species *R. ridibunda* and *R. lessonae*. In subsequent experiments Berger confirmed this finding and showed that both male and female hybrids are produced in these crossings (Berger 1968, 1970). Unlike many hybrid taxa, which are usually less viable than the parental species, *R. esculenta* proved to be ecologically very successful, occurring in most regions where the parental species are present and even beyond. This ecological and geographical success seems to be associated with its special mode of reproduction, i.e., hybridogenesis. Hybridogenesis was first discovered in fishes of the genera *Poeciliopsis* by Schultz (1969). It describes the exclusion of one parental genome during gametogenesis prior to meiosis and the propagation of only one genome to the next generation without recombination. By subsequently mating with the respective parental species, hybridogens regain the excluded genome to produce hybrid offspring again. This dependency of the hybrid on at least one parental species requires mixed population systems, and these hybridogens can be regarded as sexually parasitizing the parental species. Since the inherited genome is not recombined and therefore clonally passed on to the offspring, this mode of reproduction is often also referred to as "hemiclonal reproduction" (Dawley 1989). The presence of hybridogenesis in water frogs was demonstrated first by Tunner (1974), using protein electrophoresis. Hybridogenesis was not only detected in several other waterfrog taxa such as *R. grafi* (Graf et al. 1977) and *R. hispanica* (Uzzell and Hotz 1979), but has also been investigated

in stick insects of the genera *Bacillus* (Mantovani and Scali 1992), salamanders of the genera *Ambystoma* (Hedges et al. 1992) and in fishes of the genera *Poeciliopsis* (Vrijenhoek 1994) and *Leuciscus* (Alves et al. 2001).

### *The Rana esculenta complex*

*R. esculenta* (genotype LR), the edible frog, is formed by natural hybridization between the lake frog *R. ridibunda* (genotype RR) and the pool frog *R. lessonae* (genotype LL). It is assumed that primary hybridization has occurred repeatedly (Graf and Polls Pelaz 1989, Guex et al. 2002) and usually happens between female *R. ridibunda* and male *R. lessonae* due to morphological and behavioral reasons (Spolsky and Uzzell 1986, Günther et al. 1991). In contrast to other hybridogenetic taxa which are all-female, *R. esculenta* is a bisexual hybrid and both males and females are reproducing via hybridogenesis. Several mixed population systems have been described throughout Europe (summarized in Plötner 2005). In Western Europe, the *lessonae/esculenta* system (LE-system) is predominant, where *R. esculenta* occurs in sympatry with *R. lessonae* and persists by backcrossing with this parental form. In nature, matings also occur between hybrids (Abt 2003), but the resulting *R. ridibunda* offspring die early during the larval phase due to the accumulation of deleterious mutations on the clonal R-genome (Semlitsch and Reyer 1992, Vorburger 2001). This process is also called Mullers ratchet (Muller 1964). The inverse pattern is found in the *ridibunda/esculenta* system (RE-system), where the hybrids exclude the R-genome prior to meiosis and sexually parasitize the sympatrically occurring *R. ridibunda*.

A specialty within the *Rana esculenta* complex are the pure hybrid population systems found mainly at the northern edge of the distribution. Such populations consisting of only individuals with hybrid genotypes have arisen in several northern areas, e.g., Northern Germany, Denmark and Sweden (Günther 1975, Ebendal 1979, Fog 1994, Rybacki 1994), despite the above mentioned necessary sympatry of the hybrid with at least one parental form. Besides diploid hybrids (LR genotype) which are also found in mixed population systems, these all-hybrid populations consist of two types of triploid individuals (LLR and LRR). Polyploidization is often found in connection with altered

reproductive modes such as parthenogenesis, gynogenesis or hybridogenesis, because it provides a way to overcome difficulties in chromosome pairing during gametogenesis (Schultz 1969, Dufresne and Hebert 1994, Otto and Whitton 2000, Vrijenhoek 2006). In pure hybrid populations of *R. esculenta*, polyploidization seems to have been an important step towards reproductive independency of the hybrid. It is assumed that in pure hybrid populations, the triploid individuals provide the premeiotically excluded genome (LLR individuals provide the L-genome, LRR individuals the R-genome), which in mixed LE- and RE-systems is delivered by the respective parental species (reviewed in Plötner 2005). However, under this assumption we would expect that parental genotypes are formed in these pure hybrid populations by matings between homotypic triploids. Thus, the question arises if these populations really are all-hybrid and, if yes, how they are maintained over time. Pure hybrid populations offer an excellent system to investigate the selective advantages of a hybrid taxon over its ancestors and to study the structure and dynamics of such a system.

*Approach to my research questions*

An ideal area to study the structure and dynamics of pure hybrid populations of *R. esculenta* is Skåne (Scania, Southern Sweden), because the district is ecologically diverse and well separated from the distribution of the parental species *R. ridibunda* and *R. lessonae*. Earlier studies have reported water frog populations in Southern Sweden and concluded that they consist of only *R. esculenta* individuals (Gislén and Kauri 1959, Ebendal 1979). However, due to the lack of appropriate molecular tools, they could not determine exactly which hybrid genotypes were present. Small sample sizes in these studies do not rule out the possibility that parental genotypes occurring in low numbers have been overlooked. Finally, the temporally and spatially limited sampling in these early studies left no hint as to the population dynamics and provided no information about possible pond-to-pond differences in genotype composition.

Therefore, the primary goal of my PhD-thesis was to thoroughly investigate pure hybrid populations on a spatial and temporal scale in order to gain closer insight into their structure and persistence. First, the ability to correctly determine the genotypes of the

water frogs occurring in this area was fundamental. Morphological methods have been used for some time to discriminate between water frog species (Berger 1967, Günther 1975), but since the morphology of *R. esculenta* hybrids is intermediate between the parental species and similar for all hybrid types, an unambiguous assignment to the different genotypes is often not possible. An alternative is offered by various molecular methods (e.g., allozyme electrophoresis, microsatellite and flow cytometry analysis), which have greatly improved in the last decade (Hotz et al. 2001, Christiansen 2005). However, most studies of this type are only based on one of these methods which, again, often left the correct assignment somewhat doubtful. I therefore compared and combined different morphological and molecular methods, and I adapted additional cytological methods to the study species (**Chapter 1**). Based on the most suitable methods, I focused in **chapter 2** on the genotype composition in different ponds in this area. On that account I sampled adult and partly also subadult frogs in several populations distributed over the region to get a reliable genotypic inventory of this region in Southern Sweden and the prevailing population compositions. Additionally, I also included samples from the other two regions in Sweden where water frogs are found in order to examine possible connections between populations systems in Sweden.

It has been shown for the mixed LE-system that habitat preference of the two water frogs are different which can lead to differences in taxa compositions among ponds (Holenweg Peter et al. 2002, Plötner 2005). Since ecological features of the ponds in Southern Sweden vary substantially, I examined if the differences in genotype compositions are associated with any ecological differences (**Chapter 3**). Besides such a spatial structuring in genotype composition (which gives some insight about current patterns), I was also interested in temporal changes and stability of population compositions. Therefore, changes in population composition were compared to changes in ecological parameters in 12 ponds over a period of three years. Temporal shifts in genotype proportions can also be influenced by differing survival probabilities, which were investigated by means of a Capture-Mark-Recapture (CMR) study (**Chapter 4**).

To investigate possible genetic mechanisms underlying the absence of some genotypes, in particular of the parental genotypes LL and RR that are expected under random mating, I artificially crossed the three main hybrid genotypes found among adults (LR, LLR and LRR) and analyzed fertilization success and hatchling ability of the resulting offspring (**Chapter 5**). Furthermore, I followed the offspring throughout larval development to examine possible genetic disadvantages of certain genotypes which could lead to selective mortality. Finally, in **chapter 6** I investigated the genotypes of offspring in nature and how their proportions shifted during development. While the genotypes occurring among the eggs allow discerning between assortative and random mating, subsequent changes in relative frequencies of tadpoles and metamorphs yield information about possible genotype-specific, environmentally induced mortality during the larval stages.

I have performed my research in close collaboration with Martina Arioli, who was concurrently doing her PhD on different questions in the same pure hybrid *R. esculenta* populations.

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## SUMMARY

The existence and persistence of pure hybridogenetic water frog populations of *Rana esculenta* is extraordinary for various reasons. First, hybridization in nature is often thought to be a maladaptive process, because hybrids usually exhibit lower fitness compared to their parents. So the successful establishment of these hybrids, along with their achievement of independence from the parental species, presents an interesting system to investigate the evolutionary potential of such populations. Second, the occurrence of polyploidization in these pure hybrid populations seems to be a key factor for their evolutionary and ecological success. When different ploidy levels are present together in population systems, they usually are reproductively independent from each other. However, in pure hybrid populations of the water frog *Rana esculenta* (genotype LR), originally a hybrid between *Rana lessonae* (LL) and *Rana ridibunda* (RR), different ploidy levels (LR, LLR, LRR) are reproductively closely linked. Triploids of both sexes and diploid males produce haploid gametes which result in diploid offspring when fusing with haploid gametes from triploid mating partners. Conversely, diploid eggs of diploid females fertilized by haploid sperm of diploid or triploid males give rise to triploid offspring. Hence ploidy can change from generation to generation. The aim of this study was to explore the composition and stability of different water frog populations, as well as demographic processes operating in them, in a well-defined region in Sweden that was assumed to consist of pure hybrid populations of *Rana esculenta*.

For such a study, it is essential to know the exact genotype of each individual. However, the previously suggested methods for genotype determination were manifold and not always precise enough to discern between the various genotypes. In **chapter 1** we therefore compare results from the most promising commonly used, non-lethal methods for genotype identification. Erythrocyte planimetry provided an accurate and fast way to distinguish between diploid and triploid adults, but was unreliable when used for tadpoles or tetraploid frogs. Flow cytometry improved the discrimination power between ploidy levels 2n, 3n and 4n, but was not exact enough for genotype

determination within a ploidy level over multiple populations. With microsatellite analyses, the common genotypes (LR, LLR, LRR) could be identified unambiguously, but the method alone proved to be inadequate for detecting unusual genotypes such as tetraploid frogs, homogenomic triploids (LLL, RRR), haploids or mosaic individuals, which were all not too rare among tadpoles and froglets. It is therefore absolutely necessary to combine microsatellite analysis with flow cytometry in order to assign the individuals (adults and tadpoles) correctly to their genotype. Based on these results we subsequently analyzed all the individuals used for this thesis with these two methods.

In **chapter 2**, we examine a total of 33 ponds in the area of Skåne and one pond in Östergötland for their genotype composition. Twelve of the 33 Skåne ponds were sampled at least twice a year over a period of 3 years (2002-2004) in order to study stability of population compositions over time. Contrary to earlier assumptions, we showed that all three main genotypes (LR, LLR and LRR) occur simultaneously in the vast majority of the investigated ponds. However, the relative proportion of single genotypes varied greatly between ponds. Low frequencies of tetraploid and mosaic animals were also present in some ponds, as well as other special genotypes. Genotypic compositions were not stable between years. In most ponds, diploid LR genotypes were becoming more frequent, while mainly LLR proportions were decreasing. Parental genotypes were absent from the adult populations, with the exception of 4 RR females. Additionally, our data show strongly skewed sex ratios: LRR and RR genotypes were mainly females, while LLR and LLRR were, to a lesser extent, skewed towards males. The newly discovered population in Östergötland was identified as a mixed population of *R. lessonae* and diploid *R. esculenta*. While this population type ("LE-system") is widespread in Central Europe, it is reported for the first time within Sweden.

After showing that most ponds are occupied by all three main genotypes (LR, LLR and LRR), but differ considerably over space and time in their compositions, we were interested in finding the causes for these differences. **Chapter 3** provides data on ecological factors potentially influencing the genotype composition in a pond. We assumed that triploid frogs exhibit ecological needs similar to the parental species

because one genome is present in double copy number. We investigated features potentially influencing both the aquatic larval stage of these frogs (water chemistry, physical parameters) and the adult life stages (pond morphology, land use, climate). In general, all *R. esculenta* types were tolerant for a wide range of physicochemical conditions. Adult genotype proportions were mainly dependent on variables describing pond habitus and not on physicochemical variables. Diploid *R. esculenta* preferred larger, wooded ponds, whereas triploid LLR hybrids were more abundant in smaller, open ponds. Changes in genotype composition over the years were not strongly related to changes in physiochemical conditions, although some influence of dissolved oxygen and temperature was noticeable.

Another possible factor for differentially structured populations is examined in **chapter 4**, namely differences in survival rates between genotypes. Twelve ponds were sampled twice a year over a period of three years (2002-2004). Frogs were identified to sex and genotype and marked individually with RFID PIT tags upon first capture. Based on this recapture data, model building and selection was performed. Model selection yielded no single candidate model for adult survival that was best supported by the data. We showed that frogs, regardless of sex and genotype, had very similar biweekly survival rates, mostly around 90 %. Therefore, differential survival of genotypes in the adult stage is not the mechanism that affects the stability of the all-hybrid system.

In **chapter 5**, we examine by means of an artificial crossing experiment the gamete production of the three main hybrid genotypes, the types of the resulting offspring and their survival. Fertilization ability was similar in all three female genotypes, but differed considerably in males. Especially diploid LR males producing simultaneously haploid and diploid sperm had very low fertilization success. Overall, the crossing experiment produced the following offspring: LR, LLR and LRR hybrids, parental genotypes (LL and RR), as well as tetraploid individuals. However, towards the end of larval development survival of the parental types among the offspring was lower compared to the hybrid genotypes. These results indicate that the absence of parental genotypes among adults

in nature is not purely due to genetic incompatibilities early in larval development, but must be explained differently.

**Chapter 6** presents data on the changes in the natural genotype composition during the larval phase. In eleven different ponds, we examined the genotypes occurring in eggs masses, tadpoles and newly metamorphosed animals. Early in development, the genotypes present in the ponds were manifold. Half of the individuals had unusual genotypes: We have found several homotypic genotypes (LL, LLL, RR and RRR), some tetraploid genotypes and also genetically mosaic individuals. The proportion of these unusual larval genotypes was clearly decreasing towards later tadpole stages until, finally, almost none of these genotypes were found among the metamorphs. Therefore, although mating seems to be random in natural ponds and unusual genotypes are initially produced, they are selected against early in development. This results in only diploid and triploid (occasionally tetraploid) hybrids surviving to the adult stage in these populations.

In **conclusion**, this study shows that Skåne is in fact an area in which only pure hybridogenetic water frog populations are found. However, the populations did vary greatly in there genotype composition and were also temporally not stable. These geographical and temporal differences could not be satisfyingly explained by ecological factors or differential survival rates in adults. We are therefore inclined to believe that changes in genotypic composition, dynamics and persistence are mainly driven by stochastic events. This conclusion is further supported by the obvious random mating occurring in nature and the strong reproductive link of the genotypes: Genomes are usually passed on from diploid to triploid animals and vice versa.

## Zusammenfassung

Die Existenz und die Langlebigkeit von reinen hybridogenetischen Populationen des Wasserfroschs *Rana esculenta* sind in vielerlei Hinsicht aussergewöhnlich. Zunächst wird die Hybridisierung in der Natur gemeinhin als ein nachteiliger Prozess angesehen, da Hybride normalerweise eine geringere Fitness als ihre Elternarten aufweisen. Demnach stellt das erfolgreiche Auftreten dieses Hybrides, zusammen mit dem Erreichen der Unabhängigkeit von den Elternarten, ein interessantes System dar, an dem das evolutionäre Potential solcher Populationen untersucht werden kann. Zweitens scheint die Polyploidisierung in diesen reinen Hybridpopulationen ein Schlüsselfaktor für ihren evolutionären und ökologischen Erfolg zu sein. Sind verschiedene Ploidiestufen innerhalb eines Systems vorhanden, so sind sie normalerweise reproduktiv unabhängig voneinander. In reinen Hybridpopulationen des durch Bastardisierung zwischen *Rana lessonae* (Genotyp LL) und *Rana ridibunda* (RR) entstandenen Wasserfrosche Rana esculenta jedoch ist die Fortpflanzung verschiedener Ploidiestufen (LR, LLR, LRR) eng miteinander verknüpft. Triploide beiderlei Geschlechts sowie diploide Männchen produzieren haploide Gameten, die zu diploiden nachkommen führen, wenn sie mit den haploiden Gameten von triploiden Geschlechtspartner verschmelzen. Umgekehrt ergeben diploide Eier von diploiden Weibchen triploide Nachkommen, wenn sie von haploiden Spermien von triploiden oder diploiden Männchen befruchtet werden. Ploidien können daher von Generation zu Generation wechseln. Das Ziel dieser Untersuchung war es, die Zusammensetzung und Stabilität verschiedener reiner Hybridpopulationen zu untersuchen sowie die zu Grunde liegenden demographischen Prozesse und Einflüsse zu analysieren. Dazu wählte ich eine gut abgegrenzte Region in Südschweden, in der das ausschliessliche Vorkommen von reinen Hybridpopulationen von *R. esculenta* vermutet wurde.

Für eine solche Untersuchung bedarf es der Kenntnis des genauen Genotyps jedes Individuums. Bis anhin sind zwar zahlreiche Methoden zur Bestimmung des Genotyps vorgeschlagen worden, jede für sich ist aber nicht präzise genug, um unter allen Umständen die vollständige Unterscheidung zwischen den Genotypen durchzuführen.

In **Kapitel 1** vergleichen wir deshalb die aussichtsreichsten gebräuchlichen nichtletalen Methoden. Dabei stellte sich die Vermessung von Erythrozyten als eine schnelle und genaue Möglichkeit heraus, um zwischen diploiden und triploiden Erwachsenen zu unterscheiden. Für Kaulquappen oder tetraploide Tieren war die Methode jedoch ungeeignet. Durchflusszytometrie erhöhte das Unterscheidungsvermögen zwischen, aber nicht innerhalb der Ploidiestufen (jedenfalls nicht über mehrere Populationen hinweg). Die Mikrosatelliten-Analyse identifizierte die häufigsten Genotypen (LR, LLR, LRR) zuverlässig, erwies sich jedoch als ungeeignet, um ungewöhnliche Genotypen wie homogenomische Triploide (LLL, RRR), haploide Tiere oder genetische Mosaikindividuen zu entdecken, welche in den frühen Entwicklungsstadien nicht selten sind. Deshalb ist es unbedingt notwendig, die Mikrosatelliten-Analyse mit der Durchflusszytometrie zu kombinieren, um den Individuen (Adulte und Kaulquappen) den korrekten Genotypen zuweisen zu können. Aufgrund dieser Erkenntnis wurden alle Individuen in dieser Arbeit mit beiden Methoden analysiert.

In **Kapitel 2** untersuchen wir insgesamt 33 Teiche in Schonen (Skåne) und einen Teich in Östergötland auf ihre genotypische Zusammensetzung. Zwölf der 33 Teiche in Schonen wurden innert 3 Jahren (2002-2004) mindestens zweimal jährlich beprobt, um die zeitliche Stabilität der Populationszusammensetzung zu untersuchen. Im Gegensatz zu früheren Annahmen konnten wir nachweisen, dass alle drei Hauptgenotypen (LR, LLR und LRR) gleichzeitig in fast allen untersuchten Teichen vorkommen. Ihre relativen Häufigkeiten unterscheiden sich jedoch beträchtlich zwischen den Teichen. Ausserdem fanden sich in gewissen Teichen auch geringe Anzahlen von Tetraploiden und genetischen Mosaiktieren, nebst anderen speziellen Genotypen. Weiter konnten wir zeigen, dass die relativen Zusammensetzungen zwischen den Jahren nicht stabil blieben. In den meisten Teichen nahm der Anteil von LR Genotypen zu, während vor allem LLR Genotypen weniger häufig zu finden waren. Elterngenotypen fehlten in den adulten Proben, mit Ausnahme von 4 RR Weibchen. Unsere Daten zeigen ausserdem ein stark asymmetrisches Geschlechtsverhältnis für einzelne Genotypen: Tiere mit LRR- und RR-Genotyp waren hauptsächlich weiblich, während Tiere LLR- und LLRR-Genotyp eine etwas weniger ausgeprägte Tendenz zu Männchen zeigten. Die neu entdeckte

Population in Östergötland wurde als gemischte Population von *R. esculenta* und *R. lessonae* identifiziert. Während dieser Populationstyp (ein sogenanntes „LE-System“) in Mitteleuropa weit verbreitet ist, konnte er für Schweden zum ersten Mal beschrieben werden.

Nachdem wir zeigen konnten, dass in den meisten Teichen alle drei Hauptgenotypen (LR, LLR und LRR) gleichzeitig, aber in stark unterschiedlichen Proportionen vorkommen, interessierten wir uns für mögliche Ursachen für diese Unterschiede. Das **Kapitel 3** befasst sich mit potentiellen ökologischen Einflüssen auf die genotypische Zusammensetzung in den Teichen. Wir nahmen an, dass triploide Tiere aufgrund des einen in doppelter Ausführung vorhandenen Genoms ähnliche ökologische Bedürfnisse aufweisen wie die entsprechende Elternart. Wir untersuchten sowohl Faktoren, die einen Einfluss auf die aquatische Lebensphase der Frösche haben können (Wasserchemie, physikalische Parameter), als auch Faktoren mit potentiellem Einfluss auf die adulten Tiere (Teichmorphologie, Landnutzung, Klima). Generell tolerierten alle *R. esculenta* Typen eine grosse Bandbreite physikalisch-chemischer Bedingungen. Die relative Häufigkeit der Genotypen wurde hauptsächlich von Parametern des Teichhabitus' beeinflusst und weniger von physikalisch-chemischen Faktoren. Diploide *R. esculenta* bevorzugten grössere, beschattete Teiche in Waldgebieten, während triploide LLR-Hybriden in kleineren Teichen und offenem Gelände eine grössere relative Häufigkeit aufwiesen. Die Veränderungen der genotypischen Zusammensetzung über die Jahre waren kaum bedingt durch physikalisch-chemische Veränderungen, obwohl tendenziell gewisse Einflüsse der Menge an gelöstem Sauerstoff und der Temperatur erkennbar waren.

Ein weiterer möglicher Grund für unterschiedlich strukturierte Populationen wird im **Kapitel 4** untersucht: Unterschiede in den Überlebensraten der einzelnen Genotypen. Hierzu wurden zwölf Teiche über einen Zeitraum von 3 Jahren (2002-2004) zweimal jährlich beprobt. Geschlecht und Genotyp der gefangenen Frösche wurden bestimmt. Bei ihrem ersten Fang wurden die Tiere individuell mit einem RFID Transponder markiert. Aufgrund der Wiederfangdaten wurden mathematische Modelle erstellt und

selektiert. In der Modellselektion erwies sich kein einzelnes Modell für das Überleben der adulten Tiere als passend für alle Teiche. Wir konnten zeigen, dass die Frösche unabhängig von Geschlecht und Genotyp sehr ähnliche Überlebenswahrscheinlichkeiten von ungefähr 90% (auf einer Basis von zwei Wochen) aufwiesen. Aus diesem Grund schliessen wir den Einfluss unterschiedlichen Überlebens der Genotypen auf die Stabilität von reinen Hybridpopulationen aus.

In **Kapitel 5** untersuchen wir mittels eines künstlichen Kreuzungsexperimentes die Gametenbildung der drei hauptsächlich vorkommenden hybriden Genotypen LR, LLR und LRR, die Typen der daraus entstehenden Nachkommen, sowie deren Überleben. Die Befruchtungsfähigkeit der Weibchen war für alle drei Genotypen ähnlich, jedoch fanden wir bei den Männchen beträchtliche Unterschiede. Vor allem diploide LR Männchen, welche gleichzeitig haploide und diploide Spermien produzierten, wiesen einen sehr tiefen Befruchtungserfolg auf. Insgesamt wiesen die aus den künstlichen Befruchtungen entstandenen Nachkommen folgende Genotypen auf: LR, LLR und LRR Hybride, elterliche Genotypen (LL und RR), sowie tetraploide Individuen. Die Elterngenotypen überlebten aber im Vergleich zu den Hybridgenotypen gegen Ende der Larvalentwicklung schlechter. Die Resultate deuten an, dass das Fehlen elterlicher Genotypen in natürlichen Populationen nicht alleine aufgrund genetischer Inkompatibilitäten während der frühen Larvalentwicklung zu erklären ist.

**Kapitel 6** zeigt, wie sich die natürliche Zusammensetzung der Genotypen in den Populationen während der larvalen Phase verändert. In elf verschiedenen Teichen haben wir die Genotypenzusammensetzung von Eiballen, Kaulquappen und frisch metamorphosierten Tieren analysiert. In der frühen Entwicklungsphase sind eine Vielzahl von Genotypen in den Teichen präsent. Die Hälfte der Individuen wies ungewöhnliche Genotypen auf: Wir haben mehrere homotypische Genotypen (LL, LLL, RR, RRR), einige tetraploide Genotypen und auch genetische Mosaiktiere gefunden. Der relative Anteil dieser ungewöhnlichen Genotypen nahm später in den Kaulquappenproben stetig ab, bis schliesslich in den Proben der frisch metamorphosierten Tiere praktisch keine mehr vorhanden waren. Obwohl also die

Paarung in den Teichen zufällig abzulaufen scheint und entsprechend ungewöhnliche Genotypen produziert werden, verschwinden diese unter dem Einfluss der natürlichen Selektion aus der Population, bis schliesslich nur noch diploide, triploide (und manchmal tetraploide) Hybriden unter den erwachsenen Tieren übrig bleiben.

**Fazit**: Diese Studie hat gezeigt, dass in Schonen tatsächlich reine Hybridpopulationen von *R. esculenta* existieren. Die genotypische Zusammensetzung dieser Populationen variierte allerdings stark und war zeitlich nicht stabil. Diese geografischen und zeitlichen Unterschiede konnten durch ökologische Faktoren oder unterschiedliche adulte Überlebensraten nicht erklärt werden. Wir sind deshalb der Meinung, dass Veränderung in der Zusammensetzung der Genotypen, sowie Dynamik und Langlebigkeit der reinen Hybridpopulationen hauptsächlich durch stochastische Einflüsse gesteuert werden. Diese Schlussfolgerung wird weiter gestärkt durch die offensichtlich zufälligen Paarungen unter natürlichen Bedingungen, sowie durch die starke reproduktive Verbindung zwischen den Genotypen: Das Erbmaterial wird normalerweise von diploiden an triploide Tiere weitergegeben und umgekehrt.

# CHAPTER 1

## Genotype determination in an all-hybrid diploid-polyploid population of *Rana esculenta*: a comparison of non-lethal methods

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## Abstract

Hybrid organisms are interesting study objects in evolutionary ecology, due to their controversially discussed importance for giving rise new species and breaking down differences between species. However, the investigation of hybrids is often complicated by problems in unambiguous identification. These arise from the fact that hybrids often exhibit intermediary phenotypic features compared to their parental species and that hybridization often goes along with polyploidy. In this study, we compare different non-lethal methods for genotype determination in an evolutionary successful hybrid taxon. We investigate pure hybrid populations of the edible frog *Rana esculenta*, consisting of diploid, triploid and tetraploid animals. Comparing classical methods for genotype determination like morphometry and erythrocyte planimetry with modern tools like flow cytometry and microsatellite analysis, we show that there is no single method suitable for all conditions. We present the advantages, limitations and drawbacks for these methods and propose the combined use of flow cytometry and microsatellite analysis for the most satisfactory results. This study is also applicable to many other hybrid systems in which the non-lethal determination of genotype is of importance.

*Keywords*: diploid, polyploid, *Rana esculenta*, hybridogenesis, morphometry, erythrocyte size, microsatellites, flow cytometry, genotype, phenotype

## Introduction

Some years ago, the view on the importance of hybridization depended on whether you were a botanist or a zoologist. Botanists viewed hybridization as a force driving speciation, a view that was backed up by the fact that most angiosperm plant species seem to have developed from a hybridization event. Zoologists, however, dismissed the formation of hybrids as an "evolutionary dead-end". In recent years, however, this opinion has changed. The role of hybridization in speciation is discussed and acknowledged also in zoology (for comprehensive reviews see e.g., Arnold 1997, Dowling and Secor 1997, Seehausen 2004).

As many hybrid organisms exhibit phenotypic features intermediate to their parental species, one of the first and most important tasks is to detect and recognize hybrid organisms. Detection difficulties might be one of the reasons why hybrid taxa are not known more in the animal kingdom, as many hybrid taxa are only detected by the combined use of chromosomal, allozyme and molecular markers (Bullini 1994). Because hybridization in eukaryotes often is accompanied by polyploidization (Dowling and Secor 1997, Soltis and Soltis 1999), hybrid identification tends to get even more difficult. Polyploidy, especially in plants, can lead to novel phenotypes (Osborn et al. 2003, Otto 2003) or, as in the case of allotriploid water frogs, produce further nuances in phenotypic and genotypic intermediacy between the parental species and their primary diploid hybrid (Günther et al. 1979). Consider two diploid parental species with genotypes AA and BB. Their primary diploid hybrid (AB) is assumed to show intermediary features between AA and BB; triploid specimen of the AAB or ABB genotype will be intermediary between AB and the parental species represented by two genomes. This will lead to a phenotype gradient of the sequence AA – AAB – AB – ABB – BB. Whereas the adult genotypes and maybe even their primary hybrid might be distinguishable by morphological features, the phenotypic overlaps might become large with the presence of polyploid organisms in the population. This is the case in the European water frog system.

The European edible frog *Rana esculenta* (LR genotype) is a hybrid which originally derived from the mating of the lake frog *Rana ridibunda* (RR) and the pool frog *Rana lessonae* (LL). Reproducing hybridogenetically, *R. esculenta* discards one half of its genome (the L- or the R-part) prior to meiosis and transmits the other part clonally (Schultz 1969, Graf and Polls Pelaz 1989). To regain the lost genome, *R. esculenta* parasitizes sexually its corresponding parental species. Unlike most of the known vertebrate hybrids, *R. esculenta* males and females are both fertile and participate in reproduction.

In Europe, *R. esculenta* is mainly found in sympatry with *R. lessonae* (forming the so-called *lessonae-esculenta* (LE-) system), but also with *R. ridibunda* (RE-system), or both (Plötner 2005). In addition, pure hybrid populations are reported mainly from the northern range limits, where triploid animals (LLR or LRR) take over the role of the parental species and provide the genome that was excluded from the diploid's germ line (Günther 1983). A fundamental problem in all of these different population systems is stability over time. Due to accumulation of deleterious mutations on the clonally transmitted genome of *R. esculenta* (a phenomenon called Muller's ratchet, Muller 1964), homotypic *R. esculenta* matings generally lead to inviable offspring instead of backcrossing to the respective parental genotype (Vorburger 2001). Among the remaining three possible mating combinations, two result in hybrid offspring (parental female x hybrid male and hybrid female x parental male) and only one in offspring of the parental species (parental female x parental male). Under random mating, numerical shifts in genotype composition are to be expected. Yet, stable populations can be attained over an evolutionary long period of time, as shown by modeling of LE-systems (Hellriegel and Reyer 2000, Reyer et al. 2004), and pure hybrid systems (Som and Reyer 2006).

To examine these different population systems and the variety common and rare genotypes and changes, respectively stability, over time, we need a reliable method for genotype determination.

Earlier investigations of European water frog communities were carried out mainly by phenotype and morphometric indices and with erythrocyte (red blood cells, RBC) size comparisons (planimetry) (Uzzell et al. 1977, Berger 1988, Fog 1994). Amphibian erythrocytes are nucleated and their size is correlated with the DNA content, so that the genome size differences between various combinations of *R. lessonae* and *R. ridibunda* genomes (differing by 16% according to Vinogradov et al. 1990, Sharbel et al. 1997) are claimed to be visible through blood cell area. In the 1970s, researchers started to use serum compatibility measures and allozyme electrophoresis in addition to morphometry and RBC planimetry (Tunner 1973, Günther et al. 1979, Uzzell and Hotz 1979, Ebendal and Uzzell 1982). From the late 1990s onward, microsatellite analysis was introduced (Garner et al. 2000, Zeisset et al. 2000, Hotz et al. 2001). Along with these basic methods, researchers have used mating call differences (Günther et al. 1991, Wycherley et al. 2001), karyotyping (Tunner and Heppich-Tunner 1991, Plötner and Klinkhardt 1992), RBC densitometry (Ogielska et al. 2004), flow cytometry (Vinogradov et al. 1990) and other methods.

The reliability and accuracy of these different methods was disputed over the years (Pagano and Joly 1999, Lodé and Pagano 2000, Schmeller et al. 2001, Ogielska et al. 2004). In this paper we compare the results yielded with different, mostly non-lethal methods for determination of ploidy and genotype: phenotypic and morphological measurements, erythrocyte size, flow cytometric analysis of RBC DNA content, microsatellite data, and allozyme electrophoresis, and we discuss their advantages and limitations.

## Methods

### *Sample collection*

A total of 3793 frogs (3184 adults, 413 juveniles, and 196 metamorphs) was collected in the years 2002-2004 from 36 ponds in a pure hybrid water frog system in Skåne, Southern Sweden. Another 40 frogs (17 adults, 23 juveniles) were caught 2004 in a recently discovered LE-system near Hannäs in Östergötland, Sweden. All Animals were

measured and weighed, and adults and large juveniles were individually marked with a RFID PIT tag (Trovan ID101, Trovan Ltd., UK). One phalanx of both fourth toes was clipped for DNA and for allozyme analysis. Tissue for DNA samples was stored in ethanol at -20°C until analysis; tissue for enzyme samples was stored at -80°C until analysis. Additionally, about 30-50µl blood was taken from a web vein with a heparinized capillary tube (70µl Micro-Hematocrit Capillary Tubes, VWR International, West Chester USA) and stored in citrate buffer (D-(+)-glucose 475 mM, Sigma G8270; trisodium citrate 40 mM, Sigma-Aldrich S4641; dimethyl sulfoxide 5%, Sigma D8418; pH 7.6) at -80°C until analysis. All frogs were released within 24 hours at their capture sites.

More than 2500 tadpoles, either obtained from artificial crossings or caught in natural populations, were sampled in addition to the frog samples mentioned above. Tadpoles were put to sleep in a solution of 5g 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, Sigma A5040) per 1l $H_2O$. The tailfin was clipped and stored in ethanol at -20°C until analysis. The heart was punctured and blood was taken with a heparinized capillary tube (70µl Micro-Hematocrit Capillary Tubes, VWR International, West Chester USA; or 20µl Microcaps, Drummond Scientific Co., Broomall USA). Blood sample handling occurred according to the adult frogs.

Finally, tissue samples of adult frogs (toe clips or whole specimen) were collected or have been provided by collaborators from pure hybrid *R. esculenta* systems in Denmark and Northern Germany, LE- and RE-systems in Poland, Latvia, Lithuania and Estonia, and from pure *R. lessonae* populations in Northern Sweden.

### *Morphology*

Morphological features were measured from 3651 Swedish water frogs (adults, juveniles and metamorphs). Body length (snout-vent length, SVL), tibia length (ti), length of first toe (digitus primus, dp), as well as length of the metatarsal tubercle (callus internus, ci) were measured using a digital sliding caliper (Absolute Digimatic, Mitutoyo Corp., Kawasaki Japan). Length of callus internus was defined as the mean of three measurements. Whenever possible, measurements were carried out on the animal's right body side. The morphological features were used to determine several ratios (e.g.,

SVL/ti, ti/ci, dp/ci) by which the different water frog genotypes have been classified in past studies (Uzzell and Hotz 1979, Rybacki and Berger 2001). The weight of the animal was measured using a spring scale (Pesola Micro-Line, Pesola AG, Baar Switzerland). As additional qualitative features, the shape of the callus internus, body colour and spot patterns were visually examined.

*Erythrocyte planimetry*

Blood smears of 278 individuals (102 females, 87 males, 58 juveniles, 31 tadpoles) were prepared on microscopic slides, air dried, and examined under a wide-field photomicroscope (Polyvar, Reichert-Jung, Vienna, Austria). To calculate erythrocyte area, digital images were made at 400x magnification of at least 6 sections of the smear, where single RBC were arranged plane and well visible (Fig. 1). In every section, the circumference of 5 different RBC was marked electronically and cell area, circumference, longest axis and cell width were calculated with the software Optimas 6.5.1 for Windows (Media Cybernetics, Silver Spring, MD, USA). Final erythrocyte values of an animal were defined as the mean of these 30 measurements.

*Flow cytometry*

One to three hours prior to analysis, frozen blood samples were thawed up on ice, vortexed, and an aliquot of 100µl was transferred into an Eppendorf tube. The remaining blood was stored in the freezer again. Under constant vigorous agitation, 220µl of rainbow trout *Salmo gairdneri* red blood cells (at $2.5x10^6$ cells/ml) were added as internal standard cells, followed by 550µl of freshly made propidium iodide nuclear isolation medium staining solution (PI-NIM), which consisted of a $Na^{2+}$ and $Mg^{2+}$ free phosphate-buffered NaCl solution (PBS; 137mM NaCl, 2.7mM KCl, 10.1mM $Na_2HPO_4$, 1.8mM $KH_2PO_4$, pH 7.4), propidium iodide (PI; 50µg/ml, Fluka 81845), octylphenyl-polyethylene glycol (Igepal® CA-630; 0.6% v/v, Sigma I8896) and RNAse A (100µg/ml, Sigma R5125). For weak tadpole samples with low RBC content, the concentrations were adjusted to 200µl blood, 250µl trout cells, and 550µl PI-NIM. Samples were incubated for 15 minutes at room temperature in darkness, followed by storage in darkness at 4°C. Transportation to analysis facilities was conducted on wet ice. Immediately prior to flow

cytometric analysis, blood samples were filtered (Swinnex® syringe-driven filter holders and 45µm nylon filters, Millipore, Billerica, MA, USA) and transferred into BD Falcon analysis tubes (BD Biosciences, San Jose, CA, USA). A FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) was used to measure fluorescence of sample blood cells excited with a 15mW 488nm Argon laser. At least 10000 events were analyzed, for low quality samples up to 100000 events. Acquisition speed was kept below 500 events per second. Data collection was performed with the software Cell Quest Pro for Macintosh (BD Biosciences, San Jose, CA, USA), data analysis of FL2-H and FL2-A values (maximum fluorescence emission and total cell fluorescence) was carried out with ModFit LT 3.1 for Macintosh (©1994-2000 Verity Software House Inc, Topsham, ME, USA). DNA index (DI) was calculated as the ratio of fitted values of frog RBC vs. trout RBC. Coefficient of variation (CV) as a measure of peak broadness was determined at mean peak heights. Flow cytometrical analyses were performed in several batches over the course of 4 years (May 2002 – March 2006) at the oncology department of the University Hospital in Lund, Sweden, and at the Zentrallabor für Durchflusszytometrie at the ETH Zürich, Switzerland.

*Microsatellite analyses*

DNA of half a toe clip was extracted using either a QIAamp® DNA mini kit (Qiagen, Hilden, Germany, for 2002 and 2003 samples), or BioSprint$^{TM}$ (Qiagen, Hilden, Germany, for 2004 samples). Of the seven primer pairs used in our study (Arioli 2007), four (Ca1b5 (Garner et al. 2000), CA1b6, Ga1a19 (Arioli 2007), and Res16 (Zeisset et al. 2000)) amplified both the L- and the R-genome and showed dosage effects that could be used for genotype assignment. Primer amplification and electrophoresis for the locus Ca1b5 was done in the ecology lab at the University of Zürich. Ca1b5 amplified in a total 10 µl reaction volume containing 50-100 ng template DNA, 0.5 U Taq DNA Polymerase with 10x buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM MgCl2, 0.01% gelatin) (Sigma D1806), 100 µM of each dNTP (Roche, Basel, Switzerland), and 0.5 µM of both forward and reverse primer. PCR was then performed using the following conditions: 3 min at 94°C, followed by 29 cycles composed of 30 s at 94°C for denaturing, 30 s of annealing at 57°C, and 30 s of extension at 72°C. We added a final extension of 5 min at 72°C and

stored the product at 4°C until electrophoresis. PCR products of the locus Ca1b5 were electrophoresed using the SEA 2000® Electrophoresis Apparatus with Spreadex® gels (Elchrom Scientific, Cham, Switzerland) and stained with SYBR® Gold nucleic acid stain (Molecular Probes Inc., Eugene, OR, USA). Alleles were scored against the M3 Marker (Elchrom Scientific, Cham, Switzerland) using the Q-EL™ 330 Digital Recording and Analysis System (Elchrom Scientific, Cham, Switzerland). The three other selected primer pairs (Ca1b6, Res16, and Ga1a19) did not work contentedly on the above system because products are run double-stranded and the occurrence of heteroduplexes complicated the accurate allele scoring. PCR amplification and genotyping of these three loci was therefore performed by Ecogenics GmbH (Zürich-Schlieren, Switzerland) using a single-stranded system (ABI PRISM® 3100, Applied Biosystems, Foster City, CA, USA) as follows: for all 2002/2003 samples, PCR amplification was performed in a 10µl reaction volume containing 10-20ng of extracted DNA, 5µl HotstarTaq master mix (Qiagen, Hilden, Germany), $ddH_2O$, and 0.5µM of forward and reverse primers each. The forward primers were fluorescently labeled with FAM (Ga1a19 and Ca1b6) and HEX (Res16). The following thermo treatment was used on a TC-412 Programmable Thermal Controller (Techne Ltd., Cambridge, UK): 35 cycles with 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. Before the first cycle, a prolonged denaturation step (95 °C for 15min) was included and the last cycle was followed by an 8 min extension at 72°C. For all 2004 samples (including samples from outside Sweden), the 10µl multiplex PCR reaction contained 10-20ng of extracted DNA, 5µl 2x QIAGEN Multiplex PCR Master Mix (Qiagen, Hilden, Germany), $ddH_2O$, and 0.75µM of forward and reverse primers each. The forward primers were fluorescently labeled with FAM (Ga1a19 and Ca1b6) and HEX (Res16). The following thermo treatment on a TC-412 Programmable Thermal Controller (Techne Ltd., Cambridge, UK) was used: 35 cycles with 95°C for 30 s, 53°C for 90 s, and 72°C for 60 s. Before the first cycle, a prolonged denaturation step (95°C for 15min) was included and the last cycle was followed by a 30 min extension at 60°C. The amplified products were diluted and mixed with formamide containing GENESCAN-500 (ROX) Size Standard (Applied Biosystems, Foster City, CA, USA), and analyzed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using

GeneScan® Analysis Software 3.7 (© 1993-2000 Applera Corp., Norwalk, CT, USA). Allele scoring and gene dosage analysis of these primer pairs (Ca1b6, Res16, and Ga1a19) were performed at the University of Zürich using Genotyper® 3.7NT for Windows (© 1993-2000 Applera Corp., Norwalk, CT, USA).

*Data and statistical analysis*

Based on the morphological features snout-vent length (SVL), tibia length (ti), digitus primus length (dp) and callus internus length (ci), the indices SVL/dp, SVL/ti, SVL/ci, ti/ci, ti/dp and dp/ci were investigated for 4 categories (adult females, adult males, juveniles and metamorphs) from 6 genotypes (LL, LLR, LR, LLRR, LRR, RR). All results were compared by discriminant analyses to the final results obtained with a combination of flow cytometry (for ploidy determination) and microsatellite analyses (for genotype determination). Only samples for which these final results were unambiguous were used. The best performing morphological index was submitted to an ANOVA to determine the influence of category, genotype, measurer, pond, and their respective interactions on the mean index values. Erythrocyte values underwent UPGMA cluster analyses to investigate their discriminatory abilities. Resulting groups were again compared to the final results as described above. The relationship between SVL and RBC size was investigated with a correlation analysis. An ANOVA was performed to investigate the influence of category, genotype, ploidy and pond on the best performing RBC value. Flow cytometry results were classified arbitrarily into four quality categories according to the criteria mentioned in Fig. 2; results of qualities 1 and 2 (after adjustment) were used for genotype assignment, quality 3 results were only used for ploidy determination, and category 4 results could not be used at all. DNA indices of 1$^{st}$ or 2$^{nd}$ quality were submitted to a discriminant analysis, comparing the resulting groups to microsatellite findings.

Statistical analyses and tree plots were performed with SAS 9.1.3 SP3 for Windows (© 2002-2003 SAS Institute Inc., Cary, NC, USA.), all other graphs were produced using SigmaPlot 2002 v8.02 for Windows (© 1986-2001 SPSS Inc., Chicago, IL, USA).

## Results

### *Morphology*

Genotypes overlapped in all morphological indices. Fig. 3 shows the overlap for index ti/ci, Table 1 provides a summary for all indices. Stepwise discriminant analysis (PROC STEPDISC, significance levels for entry/stay p=0.05) showed that the index ti/ci entered the model always as first variable for all categories (metamorphs, juveniles, females and males). The next included index varied according to the category: For juveniles and females it was SVL/ti, for metamorphs and males it was SVL/dp (Table 2). The index ti/dp was not included in any model. An ANOVA for ti/ci (PROC GLM, post-hoc Scheffé's multiple comparison procedure) showed that while the index means differed significantly between all categories, there was no significant genotype*category interaction when comparing only males and females ($F_{(5)}$=2.05, p=0.07). There were, however, significant interactions between genotype and category when including also juveniles and metamorphs, as well as between genotype*measurer and genotype*pond in any case (all p<0.0001). Morphological indices of repeatedly caught animals sometimes showed considerable variation. For all measurers and/or time differences between catching events, there were mean differences of 3-5%, maximum differences between measurements reached 10-33% (Table 3). Repeated measurements within and between measuring persons showed mean differences of 2-4%, with maxima of 7-9% (Table 4). A discriminant analysis based on the indices ti/ ci, SVL/ti, SVL/ci, SVL/dp and dp/ci led to mean error rates in assignment of an individual to a genotype between 24.1% (adult males and females combined, 6 genotypes) and 41.5% for metamorphs (4 genotypes) (Table 5). Misassignment of triploid animals was mostly into LR (data not shown).

Additionally, 2566 animals were classified visually into genotypes by the researchers in the years 2002-2004, using size and shape of callus internus (Günther 1990), body color, and sometimes additional cues such as odor. This resulted in a misclassification of 1110 individuals (43.3%). Taking the last year (2004) of the investigations only to account for a possible "training effect", still 42.1% of 1292 animals were misclassified, with no

apparent differences between researchers (MA: 41.1%, n=443; CJ: 42.5%, n=849). Misassignment of triploid animals was again predominantly into LR (data not shown).

*Erythrocyte planimetry*

Of the four RBC measures (length, width, circumference and area), circumference showed best discriminatory power, based on the least misassignments: When performing separate UPGMA cluster analyses for tadpoles, juveniles, females and males, a clear separation into two distinct size groups was obtained (Fig. 4 showing the exemplary result for males). These size groups corresponded to diploid (small RBC sizes) and to polyploid, i.e., triploid and tetraploid animals (large RBC sizes, see Fig. 5). There was no further discrimination possible, neither between the different diploid or triploid genotypes, respectively, nor between tri- and tetraploid animals, although the latter showed a tendency for the largest values. The only misassignments observed were for 2 tadpoles with LL genotype which both showed large, triploid-like erythrocyte circumferences, and for 2 tadpoles with LLR and LLR genotypes, respectively (see Table 6). Both triploid animals were correctly assigned by UPGMA cluster analysis, however, and only misclassified by the discriminant analysis (with posterior probabilities of 0.52 and 0.72 to be classified as diploid). Although in most cases a trend for a positive relationship between SVL and RBC circumference was observed (and in the case of polyploid juveniles even a negative trend), the correlation between SVL and RBC circumference was significant for polyploid females only (Pearson correlation coefficient r=0.2853, n=52, p=0.04). Neither RBC area nor RBC width correlated with the SVL of the animals, however. Likewise, there was no correlation between tadpole stage and RBC values (see Table 7) when removing both LL tadpoles showing triploid-like erythrocyte values from the sample.

When using the approximate formula for ellipse circumference calculation $2\pi\sqrt{\frac{a^2+b^2}{2}}$ as an estimate to erythrocyte circumference (as low-tech substitute for automated calculations performed by optical recognition programs), the results of the discriminant analysis did not change for males, females and juveniles, but for tadpoles, there was only one misclassification of a polyploid instead of two, in addition to the two

LL genotypes mentioned above. An ANOVA for erythrocyte circumference, grouped for diploid and polyploid animals, showed that mean values differed significantly between males, females, juveniles and tadpoles (diploid: $F=299.47$, $df=3$, $p<0.0001$; polyploid: $F=454.61$, $df=3$, $p<0.0001$). Scheffé's multiple comparison procedure for group means showed that for diploid animals, RBC circumference (means of 30 cells) were significantly lower for tadpoles, but that there were no significant differences between males, females and juveniles. In polyploids, females and males, as well as females and juveniles, showed no significant differences. Finally, an ANOVA for RBC circumference, grouped by category and ploidy level, revealed significant differences between ponds.

*Flow cytometry*

Of 3499 tested adult individuals, 13 samples were assigned to category 4 (unusable quality), and 35 to category 3 (ploidy level inferable). These were mostly analyzed in 2002, when the whole blood sample was used in one analysis, whereas in 2003 and 2004, there was enough blood left for a second or third analysis in case of handling errors. 97 samples of totally 2635 (3.7%) were re-analyzed in 2003/2004. Pooled DNA indices (DI) resulting from comparison of adult frog blood samples with a trout standard showed a clear separation between di-, tri- and tetraploid animals, whereas an apparent overlap between the triploid genotypes LLR and LRR was found (Fig. 6). Mean genotype DI values varied significantly between analysis periods, ponds, and categories (Tables 8 and 9). When performing a discriminant analysis of DI values, grouped by analysis period, pond and sex, 12 of 3018 samples (0.4%) were assigned into a different genotype than expected from microsatellite data when analyzing only unambiguous samples (i.e., no tetraploid animals, mosaic, or indefinite microsatellite results). These deviant results stemmed from triploid female animals only: 3 were classified as LRR by flow cytometry and LLR by microsatellite analysis, 9 were classified as LLR and LRR, respectively.

*Microsatellite analyses*

Genotype assignment based on microsatellite data may be done by counting the number of genome-specific alleles (e.g., 1 L-allele and 2 R-alleles = LRR genotype). This

requires a high allelic diversity. Alternatively, dosage effects can be used. This method is based on the assumption that the genome present in higher frequency will be amplified more during PCR according to its ratio in which it is present compared to the other genome. If analysis yields a ratio of 2:1 for L:R peak heights, the animal is considered an LLR triploid. Not all microsatellite loci show dosage effects, however. Fig. 7 shows an example of microsatellite analysis of an LRR individual.

Allelic diversity of the *R. esculenta* populations in southern Sweden was very low for the loci under investigation (Arioli 2007); all four loci showed one L-specific allele only, whereas two (Ca1b5), respectively three R-specific alleles (Ca1b6, Ga1a19, Res16) were detected. Allelic diversity increased outside southern Sweden for all primers except Ca1b5, especially for R-specific loci (see Table 10). Out of 4597 post-metamorph individual tissue samples, 51 yielded no result for Ca1b5, seven for Ca1b6, five for Ga1a19, and eight for Res16. Because of insufficient quality, technical failures or confirmation of unusual or contradictory results, 671 samples (14.6%) had to be re-analyzed for Ca1b5, as well as 413 samples (4.6%) for Ca1b6, 222 (4.8%) for Ga1a19 and 233 (5.1%) for Res16. 112 DNA samples (2.4%) had to be re-extracted, 47 of these extracts were successfully taken from blood samples collected for flow cytometry. Out of 1934 tadpole tissue samples analyzed on the Elchrom system, 59 (3.1%) did not yield a result for Ca1b5, 24 samples (1.2%) had to be re-extracted, and 336 (17.4%) had to be re-analyzed. Out of 829 tadpole tissue samples analyzed on the ABI system, three yielded no results for Ca1b6, seven for Ga1a19, and three for Res 16. 69 samples (8.2%) had to be re-analyzed for Ca1b6, 108 (13.0%) for Ga1a19 and 84 (10.1%) for Res16. Re-analysis led to a change in genotype in 76 cases. Manual correction of automated genotype assignment (by peak height ratio of L- and R-alleles or number of alleles) changed genotype in 186 additional cases. Of 3278 samples which underwent also flow cytometric analysis, 59 showed one or more missing alleles leading to a false genotype or ploidy, 36 diploid samples were assigned as triploid by one or multiple primers. 37 triploid samples yielded contradictory results between different microsatellite primers (LLR vs. LRR), in 12 triploid cases, the assigned genotype did not correspond with flow data. No tetraploid or mosaic animals could be detected with microsatellite primers. In

29 cases, single primers suggested asymmetric tetraploid animals of LLLR or LRRR type, which was not supported by DNA index data.

The primer Ca1b6 could not be used for dosage effects for the first batch analyzed (2002/2003 samples); only in the second analysis batch were dosage effects between the L- and the R- alleles interpretable.

## Discussion

For scientific research, especially with hybrid species exhibiting several ploidy levels and showing intermediate traits, it is often essential to know the exact genotype of an individual. Since the beginning of systematic classifications, several procedures for genotype determination have been used. We have investigated some of these methods on the European water frog complex and have found distinct differences in applicability, dependability, expenditure and cost.

### *Morphology*

Morphological differences are among the oldest indicators for the discrimination of species. Phenotypic plasticity and the intermediacy of parental features in hybrid organisms leave this way of species discrimination heavily disputed (e.g., Pagano and Joly 1999). In the *R. esculenta* complex, morphological features and indices were (and sometimes still are) used to discriminate between genotypes. In accordance with earlier studies (Plötner et al. 1994), we have found that morphometric differences allowed a clear separation of the parental genotypes LL and RR (*R. lessonae* and *R. ridibunda*; although these genotypes were rare in our samples), but not between the hybrid *R. esculenta* genotypes, where overlaps are substantial. In pure *esculenta*-populations, the genotypes that are expected to be present in highest frequencies are LR, LLR and LRR, which show the least discriminatory morphological differences. Although the index means differ significantly between these genotypes (as found with an ANOVA, because of large sample sizes), intra-genotypic variation is too large to allow unambiguous assignment of individuals. The observed error rates between 24% and 42% for

morphometric indices can be attributed to phenotypic plasticity, but also to measuring errors within and between measuring persons. These errors interact when different measures are combined for morphological indices. Especially the values of the callus internus length were subject to large variation due to its small proportions, although they were averaged over three measurements. Because of further interactions between genotype and pond, as well as genotype and sex, morphological indices should, if necessary at all, only be compared between animals of the same pond, of the same sex (or age class, if sex is not distinguishable, but note that females have constantly higher index values than males), and measured by the same person. Under these circumstances it is doubtful that standard sampling schemes will yield large enough sample sizes for statistical analysis. The fact that not all genotypes are present in all ponds (Jakob et al., chapter 2 in this publication) makes the distinction even more difficult. At last, an obvious objection against morphometry: it can only be used on animals that display distinct morphological features at all, so it is inapplicable for eggs, embryos and tadpoles, on animals with distorted growth, or on adults with mutilated extremities (e.g., through predation, frostbite).

When classifying animals by visual cues only, an error rate of about 42% occurred, which did not improve with the amount of experience of the investigators, nor was there a significant difference between researchers. This result advises to treat anecdotic references to genotypic compositions of *R. esculenta* populations based on visual characteristics (Ebendal 1979, Günther 1990, Plötner 2005) with caution.

*RBC size*

Like morphological features, RBC size is a fast, simple, and therefore cheap indicator that can be used directly in the field, so it was used extensively (Berger 1988, Fog 1994, Rybacki and Berger 2001, Schmeller et al. 2001, Christiansen 2005). Unlike earlier publications that have used RBC area, we have found the circumference value to have highest discriminatory power. We have shown that RBC circumference is a highly accurate measure of di- or polyploidy, but cannot discriminate with a higher resolution. Amphibian erythrocyte attributes can vary according to SVL (resp. weight), sex, age,

DNA content, season and a variety of environmental factors (Glomski et al. 1997, Schmeller et al. 2001). We could show the necessity of a classification into at least 3 age classes: adult frogs, juveniles, and tadpoles; whenever possible, the adult sexes should be separated, too. We have shown that inter-pond differences in RBC size are significant, but the difference between diploid and polyploid RBC size was large enough to still deliver accurate results when pooling all ponds. However, direct size comparisons between different ponds are not possible. In spite of the aforementioned advantages of RBC planimetry, there are several handicaps to this method: first, it cannot be used for early developmental stages lacking erythrocyte development. Second, it cannot be used to differentiate between tri- and tetraploid animals, nor between genotypes within a ploidy level. And third, the occurrence of apparently triploid sized erythrocytes within diploid animals is problematic in the sense that it concerned LL-genotypes. Most other methods that are used cannot distinguish between diploid LL and autopolyploid animals like LLL etc. In such cases, the sole use of RBC values (Blommers-Schlösser 1990, Berger and Berger 1994) may lead to misclassifications. This may explain the apparent findings of adult LLL animals in the aforementioned reports, although they are not expected to survive (Berger 1988).

*Flow Cytometry*

Flow cytometry is a fast, reliable, and cheap method (Murphy et al. 1997), and is used in a variety of investigations on amphibian population compositions, (e.g., Tank et al. 1987, Vinogradov et al. 1990, Sharbel et al. 1997, Cavallo et al. 2002, Stöck et al. 2002, Lampert et al. 2003, Ogielska et al. 2004, Ramsden et al. 2006). There was considerable variation in mean DI values between analyzing phases, and between different flow cytometers, therefore analysis of all samples of a given population within one day is recommended and direct DI comparisons should be made with caution (see Fisher et al. 1994, Murphy et al. 1997). Compared to the mean diploid LR DI value, female LL blood cells contain 94% DNA, RR 108.4%, LLR 142%, LRR 148.4%, and LLRR 180.8%. Males have consistently 1-5% higher mean DI values than females, which could perhaps be used for a rough sex determination in subadult animals, but would be subject to further

experiments. Sex determination by flow cytometry is also known from other vertebrates, e.g., birds (Nakamura et al. 1990, Svensson and Nilsson 1996).

In our experiments, we have used rainbow trout erythrocyte cells as internal standard, because these were obtainable easily in large quantities and cheap. For determination of exact DNA content rather than genotype, a standard with similar cDNA content as diploid *R. esculenta* should be used, preferably also a frog species (Tiersch et al. 1989, Murphy et al. 1997), e.g., *Xenopus* or other *Rana* species. Alternatively, commercially available standards can be used, which increases however the average costs of sample analyses.

Whenever possible, blood samples should be used for flow cytometry, because the resulting peaks have low coefficients of variation (CV) and therefore allow for more accurate measures compared to other tissues (Tiersch and Wachtel 1993, Murphy et al. 1997). Because the DI values of blood are substantially different to those from other tissues, they cannot be compared directly and a mixture of blood and other tissues will lead to broader peaks and could account for false detection of aneuploidy or mosaicism (Tiersch and Wachtel 1993). This limits the use of flow cytometry for accurate genotype determination to later tadpole stages, when blood cells are present in a sufficient amount. Successful DI results could be obtained from tadpoles at Gosner stage 36 or later (Gosner 1960). Flow cytometry can be performed with tissue samples also and allows unambiguous ploidy determination, but resulting DI peaks have a high CV and are not suitable to compare between genotypes within a ploidy level (data not shown). High CV values can also indicate the influence of genotoxic environmental effects (Tiersch and Wachtel 1993, Lowcock et al. 1997, Bihari et al. 2003, Matson et al. 2004). However, in the samples analyzed for this study, no unusual high CV could be found other than in samples of quality 3 and 4, which could be attributed to problems in sampling, preparation, or storage.

As the only method discussed in this investigation, flow cytometry also identified mosaic animals, tetraploid LLRR animals, homogenetic triploid (LLL) tadpoles, and

somatic haploid tadpoles. Average sample costs were 1.36 SFr. When calculating with 3.7% necessary re-analyses, this amounts to 1.41 SFr per sample.

*Microsatellites and enzyme electrophoresis*

Protein electrophoresis was used predominantly in the *R. esculenta* system in the past for genotypic analysis (Tunner 1973, Günther et al. 1979, Ebendal and Uzzell 1982, Blommers-Schlösser 1990, Günther et al. 1991, Plötner and Klinkhardt 1992, Fog 1994, Pagano et al. 1997, Pagano and Joly 1999, Vorburger 2001, Guex et al. 2002). When investigating the use of protein electrophoresis for our study based on LDH-B and MPI, 32 of 70 samples (45.7%) were either classified wrongly, showed indeterminable patterns or did not yield a result at all (data not shown). In another test, the results were similar (U. Reyer, pers. comm.). Most probably, this can be attributed to too little suitable tissue provided from a clipped phalanx. For non-lethal and relatively harmless investigations on *R. esculenta* populations, allozyme electrophoresis is therefore not applicable, since most allozyme electrophoresis used muscle, liver or gonadial tissue (Uzzell and Hotz 1979, Günther et al. 1991, Sjögren Gulve 1991, Pagano et al. 1997).

Microsatellite analysis has the advantage of higher variability and therefore higher resolution for population genetics (Rowe et al. 1999, Hotz et al. 2001) and only a small amount of any type of nucleated tissue is needed. In the case of amphibians, clipped toes can be replaced by blood as shown in our case, or by buccal swabs (Pidancier et al. 2003, Poschadel and Möller 2004) to reduce the harming of the animal to a minimum. The use of microsatellites in population genetic analysis has become a standard over the past decade (Selkoe and Toonen 2006). In the case of hybrid animals, microsatellites can provide information about the genotype, combined with ploidy determination methods like flow cytometry, as in *Poecilia*, *Squalius*, and *Ambystoma* (Lampert et al. 2005, Pala and Coelho 2005, Ramsden et al. 2006). It has been proposed recently that microsatellite analysis can be used also for simultaneous determination of ploidy and genotype in diploid and triploid water frogs (Christiansen 2005, Christiansen et al. 2005). This can be achieved by either highly polymorphic genome-specific primers, a

large number of less polymorphic genome-specific primers, or primers which show dosage effects.

When using microsatellite analysis on 3278 Swedish samples, 37 samples showed disagreeing patterns of triploid genotypes (see Fig. 8), whereas 95 samples displayed additional or lost alleles in some primers (Fig. 9). Christiansen et al. (2005) suggested that disagreeing patterns of proposed genotypes between different primers are indicators for aneuploid animals. Based on flow cytometric data, we would instead support the findings of Ogielska et al. (2004), who showed by karyotyping that *R. esculenta* from Poland deviate in most cases from the expected parental chromosome numbers (13 chromosome per single genome each), but are nonetheless all euploid (possessing 26 or 39 chromosomes in total). This is also supported by our flow cytometry results: neither CV nor DI showed unusual values expected from aneuploid animals. Under this assumption, the number of contradictory results will likely increase with the number of primers that are used.

Because of the low genetic variability of pure hybrid water frog populations in southern Sweden (Arioli 2007), there is a very low chance to detect symmetric tetraploid animals (LLRR), because gene dosage delivers a diploid-like LR signal (see Fig. 10), and heterozygote animals are rare. The same problem applies to homogenomic triploid animals (LLL, RRR), or to somatic haploids (L, R), as shown in Fig. 11. Indications for asymmetric tetraploids (LLLR, LRRR) given by amplification patterns of L:R in 3:1 or 1:3 ratio were all shown to be artefacts and were unambiguously attributed to triploid animals by flow cytometry. Mosaic animals were not detected by the microsatellite approach (c.f. Fig. 12). This could be attributed to the fact that tissue samples were taken at one locally confined spot of the body, whereas mosaic water frogs are expected to have patchy distribution of mosaic tissues over their body (Berger and Ogielska 1994). All this requires flow cytometry as an additional method for genotype identification.

Some problems that have to be faced when analyzing PCR-based products, especially when multiplexing different primers, are unequal amplification of alleles within and

between samples because of short allele dominance, unequal amounts of template DNA, unequal primer amplification efficiency, and allelic dropouts (see reviews by Bonin et al. 2004, Pompanon et al. 2005). This can lead to allele height ratios that do not reflect the true genotype. In the case of too much template DNA, band intensities of alleles (reflected in peak height) can "overshoot" off-scale, leading on the one hand to broad, cut-off peaks returning inaccurate peak heights and therefore falsifying height ratios. On the other hand, too much template DNA can, at least in the case of the ABI system, also lead to "compensatory peaks" leaking into other colour lanes, which may influence automated labelling and result in artefact alleles (Fig. 13). In the case of short allele dominance, peaks of long alleles will be relatively smaller than peaks of shorter alleles of the same genome copy number; hence, they will lead to a different peak height ratio. This has to be accounted for by a correction factor if the alleles are differing in length by a large number of base pairs (see Fig. 14). When different alleles are situated close to one another, stutter bands can interfere with actual allele peaks through signal addition. Visual interpretation of such cases can be arbitrary and subjective. Unequal primer efficiency can lead to differential relative amplification, especially in early (annealing) phases (Mackay et al. 2002) of the individual primer's amplification curve. The inherent presence of an internal standard in the case of primers amplifying both genomes (L and R) of hybrids mitigates the impact of the late PCR stage (plateau phase), however (Morrison and Gannon 1994). Allelic dropout is considered to be a stochastic sampling error caused by low template DNA amounts (Taberlet et al. 1996, Taberlet and Luikart 1999). The larger the allele is, the higher the probability of allelic dropouts gets, but size was shown not to be the only factor (Buchan et al. 2005). Other reasons for allelic loss in the case of hybrids are: aneuploidy, introgression of DNA (substitution of e.g., L-genes or chromosomes by R genome, see Tunner and Heppich 1981, Uzzell 1982, Mezhzherin and Morozov-Leonov 1997, Schmeller 2004), mutation of primer binding sites, and methylation. An unexpected and not yet explicable error encountered in this study was the consistent presence of false signals throughout several plates (see Fig. 15), which were not attributable to PCR contamination.

It has been shown by this study that alleles can be assigned to specific parental genomes and the allele ranges of parental genomes are well separated in Swedish populations for the loci under investigation. However, in most European water frog populations, allelic diversity is higher than in Sweden, and allele size ranges between L and R specific alleles can overlap (Arioli 2007): for Res16, a single L-specific allele is known from Sweden (124bp), which is consistently outside the range for R-specific alleles (126-132bp), other than in Denmark, where L-alleles of 128bp length have been found, or in Poland, where R-allele size range starts at 122bp. Additionally, some populations have been shown to possess a large allele size range: for the microsatellite primer Ga1a19, the R-specific alleles range from 201-207bp in Sweden, but from 201-253bp in Poland (Arioli 2007). This has direct consequences for multiplexing approaches, as possible allele ranges between different loci must not overlap. Additionally, the possibility to miss unusually long alleles for other reasons is high.

As a conclusion, unusual or contradictory results have to be confirmed by re-analysis (Pompanon et al. 2005) and if persistent, compared to results obtained with other methods like flow cytometry. This may significantly increase average sample costs for microsatellite analysis, which are already quite substantial: Average costs (without DNA extraction) ranged between 1.60 SFr (single primer) and 3.49 SFr (3 multiplexed primers) for adult samples (including re-analyses) and between 1.62 SFr (single primer) and 3.66 SFr (3 multiplexed primers) for tadpole samples (see Table 11 for details). Average DNA extraction cost amounted to 4.46 SFr per tissue sample. Including all necessary re-extractions, DNA extraction of a post-larval tissue sample cost 4.57 SFr, for tadpole tissue 4.51 SFr. Whereas DNA extraction cost per tissue sample are fixed, average sample costs can be lowered by using multiplex approaches. Up to 9 microsatellite primers are multiplexed simultaneously in our lab in another project (S. Röthlisberger, pers. comm.) for use on the ABI system. For practical reasons, multiplex approaches are not possible on the Elchrom system with the primers in use.

## Conclusions

We could show that of the "classic methods", morphometry is unreliable for genotype determination of water frog hybrids. Erythrocyte planimetry, however, provides an accurate way to distinguish between di- and polyploid animals, but has its limitations for tadpoles and fails to accurately distinguish between different levels of polyploidy. We found that microsatellite analysis can deliver not only valuable information about population genetics, but is also in many cases useful for ploidy determination. However, the method was unsuitable to detect cases like tetraploid LLRR, homogenomic triploids, haploids, or mosaic animals from our samples. These special genotypes are present only in low frequencies among adult animals (Jakob et al., chapter 2 in this publication). Hence, they may be considered to be negligible as proposed by Christiansen et al. (2005) and therefore, microsatellites may be considered a suitable tool to discern between genotypes in pure hybrid populations. But this conclusion is premature, as the actual role of tetraploid *R. esculenta* has yet to be investigated. In the *Squalius alburnoides* complex, for example, tetraploid hybrids are considered to be stepping stones towards speciation, because they allow for normal meiosis and recombination to be reintroduced into the system (Alves et al. 2001, Pala and Coelho 2005). It has also been shown that amphidiploidy recovers the viability of hybrids of European and East Asian water frogs (Ohtani et al. 1997).

In water frog tadpoles (both resulting from natural and from artificial crossings), unusual genotypes are common, however (Jakob and Arioli, chapter 5 in this publication, Arioli and Jakob, chapter 6 in this publication), therefore flow cytometry, or any other reliable ploidy determination method, is necessary, as microsatellite analysis alone will not yield exact results when the number of alleles per microsatellite primer set is small.

Finally, when money is a key issue or when genetic data are not needed, microsatellite analyses can be left out completely for genotypic analyses in favour of flow cytometry, as this method did even deliver more detailed results in the course of this study.

Ramsden et al. (2006) have published a similar paper to this work, where different methods of ploidy and genotype determination in sexual and sympatric unisexual *Ambystoma*, namely isozyme identification, microsatellite identification and flow cytometric analysis, are compared. In their case, microsatellites have no disadvantage compared to flow cytometric analysis, mainly because the allele numbers of the microsatellite primer sets in use are much higher (up to 16 per set and parental species), so that their resolution is much higher.

## Acknowledgements

We would like to thank Sandra Röthlisberger for help with the lab work, the group of Rolf Zinkernagel at Zürich University Hospital for providing us access to their FACSCalibur, Bo Baldetorp at Lund University Hospital for introduction to and training in flow cytometry protocols, Hansjürg Hotz and Gaston Guex for support with allozymes, Trent J. Garner for help in microsatellite evaluation, and for providing us with unpublished microsatellite primer data. Uli Reyer, Mariusz Rybacki, Anssi Laurila and Ditte G. Christiansen kindly provided additional tissue samples. Alexandre Gouskov carried out measurements of RBC area. Finally, Uli Reyer provided helpful comments on the manuscript.

The work was funded by the Swiss National Science Foundation, grant No. 3100-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-18591-02, 522-9286-03, 522-6571-04) and by Länsstyrelsen Östergötland (522-1923-04).

## Author contributions

C.J. and M.A. contributed equally to this work. Both authors carried out all field- and lab work together. CJ performed statistical analyses and wrote the paper. Both authors discussed the results and MA commented on the manuscript.

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## Tables

**Table 1:** Summary table of morphological indices for metamorphs (MM, newly metamorphosed animals), juveniles (Juv, subadults, after first hibernation), adult females (F) and adult males (M). Indices are calculated from SVL (snout-vent length), ti (tibia length), dp (digitus primus length), and ci (callus internus length). SD=standard deviation.

| | | SVL/ci | | SVL/ti | | SVL/dp | | ti/ci | | ti/dp | | dp/ci | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Genotype | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | n |
| MM | LLR | 15.93 | 1.47 | 2.14 | 0.14 | 7.43 | 0.65 | 7.43 | 0.47 | 3.47 | 0.22 | 2.15 | 0.19 | 42 |
| | LR | 16.11 | 1.23 | 2.14 | 0.11 | 7.29 | 0.59 | 7.54 | 0.55 | 3.41 | 0.2 | 2.22 | 0.22 | 107 |
| | LRR | 17.5 | 1.27 | 2.05 | 0.11 | 6.79 | 0.61 | 8.57 | 0.64 | 3.32 | 0.21 | 2.59 | 0.22 | 41 |
| | RR | 17.18 | - | 1.99 | - | 6.61 | - | 8.64 | - | 3.32 | - | 2.6 | - | 1 |
| Juv | LL | 14.65 | 1 | 2.22 | 0.1 | 7.84 | 0.61 | 6.61 | 0.19 | 3.55 | 0.39 | 1.88 | 0.23 | 7 |
| | LLR | 15.5 | 1.06 | 2.12 | 0.09 | 7.51 | 0.47 | 7.3 | 0.41 | 3.54 | 0.16 | 2.07 | 0.14 | 70 |
| | LR | 16.25 | 1.29 | 2.1 | 0.09 | 7.37 | 0.53 | 7.74 | 0.51 | 3.51 | 0.21 | 2.21 | 0.21 | 232 |
| | LLRR | 15.69 | - | 1.92 | - | 6.12 | - | 8.16 | - | 3.18 | - | 2.56 | - | 1 |
| | LRR | 17.93 | 1.49 | 2.04 | 0.09 | 7.12 | 0.53 | 8.8 | 0.6 | 3.49 | 0.21 | 2.53 | 0.23 | 92 |
| F | LL | 13.64 | 1.33 | 2.3 | 0.09 | 7.73 | 0.37 | 5.92 | 0.42 | 3.36 | 0.13 | 1.77 | 0.17 | 5 |
| | LLR | 14.92 | 1.14 | 2.19 | 0.09 | 7.81 | 0.42 | 6.81 | 0.49 | 3.56 | 0.18 | 1.91 | 0.17 | 335 |
| | LR | 15.79 | 1.12 | 2.15 | 0.09 | 7.64 | 0.48 | 7.33 | 0.5 | 3.55 | 0.22 | 2.07 | 0.18 | 641 |
| | LLRR | 16.95 | 0.96 | 2.1 | 0.08 | 7.35 | 0.3 | 8.09 | 0.24 | 3.51 | 0.23 | 2.31 | 0.21 | 3 |
| | LRR | 17.51 | 1.44 | 2.07 | 0.1 | 7.27 | 0.48 | 8.45 | 0.62 | 3.51 | 0.2 | 2.42 | 0.23 | 745 |
| | RR | 23.98 | 1.84 | 1.93 | 0.06 | 6.67 | 0.2 | 12.43 | 0.8 | 3.46 | 0.14 | 3.6 | 0.36 | 4 |
| M | LL | 13.44 | 0.8 | 2.29 | 0.09 | 7.64 | 0.45 | 5.88 | 0.59 | 3.34 | 0.06 | 1.77 | 0.21 | 2 |
| | LLR | 14.37 | 0.99 | 2.13 | 0.08 | 7.61 | 0.44 | 6.75 | 0.43 | 3.58 | 0.2 | 1.89 | 0.15 | 515 |
| | LR | 15.21 | 1.01 | 2.09 | 0.08 | 7.46 | 0.4 | 7.27 | 0.45 | 3.57 | 0.18 | 2.04 | 0.16 | 633 |
| | LLRR | 15.39 | 0.59 | 1.99 | 0.05 | 6.91 | 0.33 | 7.72 | 0.16 | 3.47 | 0.15 | 2.23 | 0.11 | 6 |
| | LRR | 17.16 | 1.37 | 2.04 | 0.07 | 7.04 | 0.48 | 8.4 | 0.61 | 3.45 | 0.21 | 2.44 | 0.2 | 43 |

**Table 2:** Results of stepwise discriminant analysis for six morphological indices, and four categories as described in Table 1. Numbers denote the step in which the index was added to the model.

| | SVL/ti | SVL/dp | SVL/ci | ti/dp | ti/ci | dp/ci |
|---|---|---|---|---|---|---|
| Metamorphs | - | 2 | - | - | 1 | - |
| Juveniles | 2 | - | 3 | - | 1 | - |
| Females | 2 | 4 | 3 | - | 1 | 5 |
| Males | 5 | 2 | 4 | - | 1 | 3 |

**Table 3:** Mean and maximum differences (rounded percentages) and standard deviation (mm) between morphological indices calculated for repeatedly caught animals (different dates). Indices as described in Table 1. N(comparisons)=306.

| | SVL/ti | SVL/dp | SVL/ci | ti/dp | ti/ci | dp/ci |
|---|---|---|---|---|---|---|
| Mean difference (%) | 2.9 | 3.4 | 4.5 | 3.3 | 3.4 | 4.2 |
| Maximum difference (%) | 10.0 | 15.8 | 28.6 | 12.3 | 22.1 | 33.1 |
| Standard deviation (mm) | 0.1 | 0.3 | 0.7 | 0.1 | 0.3 | 0.1 |

**Table 4:** Mean and maximum differences (rounded percentages) and standard deviation (mm) between morphological measurements carried out repeatedly on the same animal (within the same day), by the same or different measuring persons. Comparisons were done for SVL (snout-vent length), ti (tibia length), dp (digitus primus length), and ci (callus internus length). Ci values were means of 3 measurements.

| | Between measurers | | | | Within measurers | | |
|---|---|---|---|---|---|---|---|
| | SVL | ti | dp | ci | ti | dp | ci |
| Mean difference (%) | 0.7 | 0.8 | 2.0 | 5.1 | N/A | 3.7 | 3.9 |
| Maximum difference (%) | 2.1 | 2.6 | 3.7 | 16.6 | N/A | 5.5 | 7.2 |
| Standard deviation (mm) | 0.5 | 0.3 | 0.2 | 0.2 | N/A | 0.3 | 0.2 |

**Table 5:** Results of the discriminant analysis using the five most powerful morphological indices ti/ci, SVL/ti, SVL/ci, SVL/dp, dp/ci. Error rates show proportion of samples of a given genotype classified wrongly. Sample sizes are shown in parentheses.

| | LL | LLR | LLRR | LR | LRR | RR | Mean |
|---|---|---|---|---|---|---|---|
| Metamorphs | - | 0.4762 | - | 0.5514 | 0.5122 | 0.0000 | 0.4146 |
| | (n=0) | (n=42) | (n=0) | (n=107) | (n=41) | (n=1) | (n=191) |
| Juveniles | 0.1429 | 0.4571 | 0.0000 | 0.4095 | 0.3370 | - | 0.2693 |
| | (n=7) | (n=70) | (n=1) | (n=232) | (n=92) | (n=0) | (n=402) |
| Females | 0.0000 | 0.4687 | 0.3333 | 0.4399 | 0.4309 | 0.0000 | 0.2788 |
| | (n=5) | (n=335) | (n=3) | (n=641) | (n=745) | (n=4) | (n=1733) |
| Males | 0.5000 | 0.2485 | 0.3333 | 0.3870 | 0.3256 | - | 0.3589 |
| | (n=2) | (n=515) | (n=6) | (n=633) | (n=43) | (n=0) | (n=1199) |
| Adults (Males + Females) | 0.0000 | 0.3518 | 0.3333 | 0.4576 | 0.3033 | 0.0000 | 0.2410 |
| | (n=7) | (n=850) | (n=9) | (n=1274) | (n=788) | (n=4) | (n=2932) |

**Table 6:** Results of the discriminant analysis carried out with erythrocyte circumference. Error rates show proportion of samples of a given ploidy class affiliated wrongly. Sample sizes are shown in parentheses.

| | Diploid | Polyploid | Total Mean |
|---|---|---|---|
| Tadpoles | 0.1429 | 0.1176 | 0.1303 |
| | (n=14) | (n=17) | (n=31) |
| Juveniles | 0.0000 | 0.0000 | 0.0000 |
| | (n=34) | (n=24) | (n=58) |
| Females | 0.0000 | 0.0000 | 0.0000 |
| | (n=50) | (n=52) | (n=102) |
| Males | 0.0000 | 0.0000 | 0.0000 |
| | (n=44) | (n=43) | (n=87) |

**Table 7:** Pearson correlation coefficients (r) of erythrocyte area, circumference and width with tadpole stage or, in the case of post-metamorphic stages, SVL. Significant p values are printed in bold. 2 outlier tadpoles were removed from the sample prior to analysis (see text).

| | | | Area | | Circumference | | Width | |
|---|---|---|---|---|---|---|---|---|
| | Ploidy | n | r | p | r | p | r | p |
| Tadpoles | Di | 12 | -0.0098 | 0.9758 | -0.1796 | 0.5765 | 0.0023 | 0.9944 |
| (stage) | Poly | 17 | -0.0651 | 0.8041 | -0.1222 | 0.6402 | -0.2094 | 0.4199 |
| Juveniles | Di | 34 | 0.1246 | 0.4826 | 0.1618 | 0.3606 | -0.0321 | 0.8572 |
| (SVL) | Poly | 24 | -0.1568 | 0.4645 | -0.1802 | 0.3996 | 0.0168 | 0.9377 |
| Females | Di | 50 | 0.2319 | 0.1051 | 0.2452 | 0.0861 | 0.1327 | 0.3585 |
| (SVL) | **Poly** | 52 | 0.1925 | 0.1716 | 0.2853 | **0.0404** | -0.0885 | 0.5325 |
| Males | Di | 44 | 0.2466 | 0.1066 | 0.2396 | 0.1173 | 0.1522 | 0.3240 |
| (SVL) | Poly | 43 | 0.1752 | 0.2611 | 0.2549 | 0.0990 | -0.0215 | 0.8912 |

**Table 8:** Pooled mean genotypic DNA indices compared to trout standard cells for tadpoles, metamorphs, juveniles, females, and males. SD=standard deviation.

| | Tadpoles | | | Metamorphs | | | Juv | | | F | | | M | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | Mean | SD | n | Mean | SD | n | Mean | SD | n | Mean | SD | n | Mean | SD | n |
| LL | 2.34 | 0.03 | 122 | - | - | 0 | 2.35 | 0.02 | 7 | 2.35 | 0.02 | 5 | 2.37 | 0.01 | 2 |
| LLR | 3.53 | 0.07 | 143 | 3.59 | 0.02 | 42 | 3.55 | 0.05 | 70 | 3.55 | 0.05 | 311 | 3.58 | 0.04 | 468 |
| LLRR | 4.49 | 0.07 | 26 | - | - - | 0 | 4.92 | - | 1 | 4.52 | 0.08 | 3 | 4.78 | 0.17 | 5 |
| LR | 2.50 | 0.04 | 308 | 2.53 | 0.02 | 106 | 2.51 | 0.02 | 229 | 2.50 | 0.03 | 604 | 2.51 | 0.02 | 588 |
| LRR | 3.67 | 0.06 | 201 | 3.75 | 0.03 | 41 | 3.72 | 0.07 | 91 | 3.71 | 0.06 | 672 | 3.74 | 0.05 | 38 |
| RR | 2.66 | 0.04 | 182 | 2.68 | - | 1 | - | - | 0 | 2.71 | 0.01 | 4 | - | - | 0 |

**Table 9:** Effects of analysis period, category (metamorphs, juveniles, females, males), and pond on mean DI per genotype, calculated by an ANOVA (PROC GLM in SAS). Significant p values < 0.05 are printed bold.

| | Period | Category | Pond |
|---|---|---|---|
| LL (n=14) | F=3.50; p>0.05 | F=2.86; p>0.05 | F=3.62; p>0.05 |
| LLR (n=891) | **F=67.64; p<0.0001** | **F=13.31; p<0.0001** | **F=2.64; p<0.0001** |
| LLRR (n=9) | **F=793.14; p<0.05** | F=8.17; p>0.05 | F=103.72; p>0.05 |
| LR (n=1527) | **F=48.12; p<0.0001** | **F=27.41; p<0.0001** | **F=2.80; p<0.0001** |
| LRR (n=842) | **F=41.81; p<0.0001** | **F=3.50; p<0.05** | **F=2.14; p<0.01** |
| RR (n=5) | - | - | F=0.57; p>0.05 |

**Table 10:** Number of different microsatellite primer alleles found in samples from Skåne (southern Sweden) and from around the Baltic Sea (northern Sweden, Denmark, northern Germany, northern Poland, western Latvia, western Lithuania, western Estonia) for L- and R- specific loci.

| | Skåne | | Around Baltic Sea | |
|---|---|---|---|---|
| Primer | L | R | L | R |
| Ca1b5 | 1 | 2 | 1 | 2 |
| Ca1b6 | 1 | 3 | 3 | 7 |
| Ga1a19 | 1 | 3 | 1 | 8 |
| Res16 | 1 | 4 | 1 | 4 |

**Table 11:** Average sample costs for microsatellite analyses per individual analysis for post-metamorphic and tadpole tissue samples (in CHF). DNA extraction costs are not included.

| | Post-metamorphic samples | | Tadpole samples |
|---|---|---|---|
| Primer | Per run | Including re-analysis | Including re-analysis |
| Ca1b5 | 1.43 | 1.60 | 1.62 |
| Ca1b6<br>Ga1a19<br>Res16 | 2.62 (individual)<br>3.18 (multiplexed) | 3.49 (multiplexed) | 3.66 (multiplexed) |

## Figures

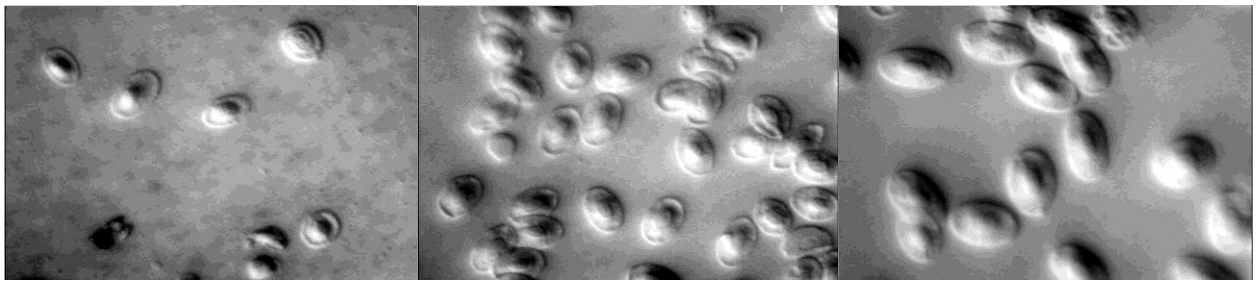

**Fig. 1:** Erythrocyte microscopic pictures of diploid tadpole (left), diploid female (middle), and triploid female (right).

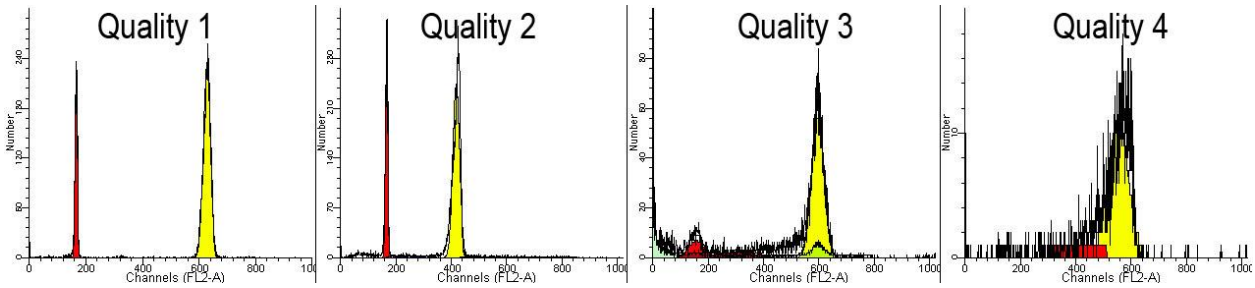


**Fig. 2:** Arbitrary quality categories for flow cytometry results: Quality 1: Exact and high peaks for both standard (left) and sample (right) peaks. Quality 2: Apparent shoulder in one peak, or peak count < 50. Quality 3: broad / low standard or sample peaks. Exact determination of DNA indices is no longer possible, but ploidy level is inferable. Quality 4: missing peaks or general bad sample quality. No information about genotype or ploidy level possible.

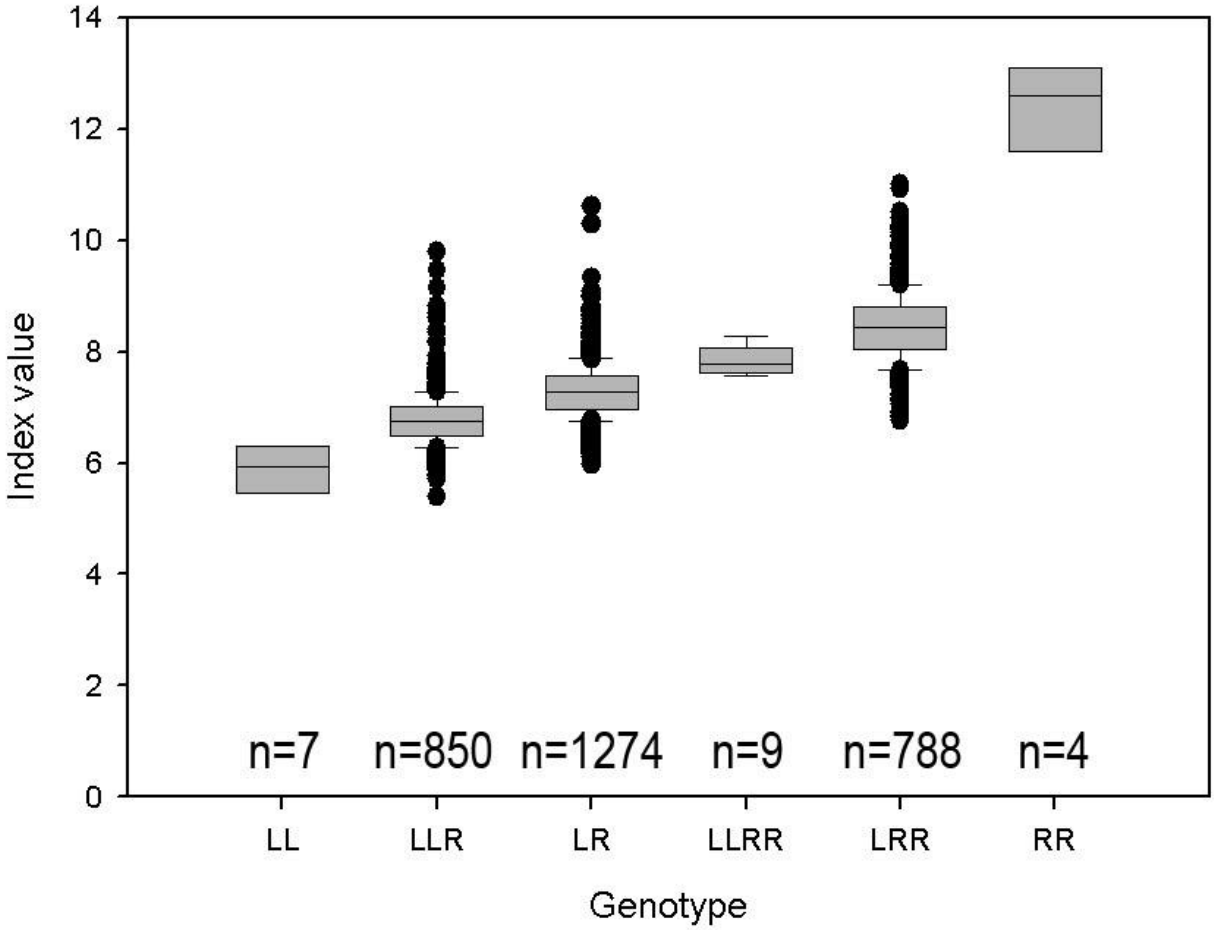


**Fig. 3:** Overlap of morphological indices (ti/ci = ratio tibia:callus internus) for six genoytpes. Ti/ci is the index best discriminating between the genotypes for all categories. Only data from adult females and males are shown. Whiskers delimit the upper and lower 95% confidence intervals, boxes are delimited by 25$^{th}$ and 75$^{th}$ percentiles, lines within boxes are medians, black dots are outliers.

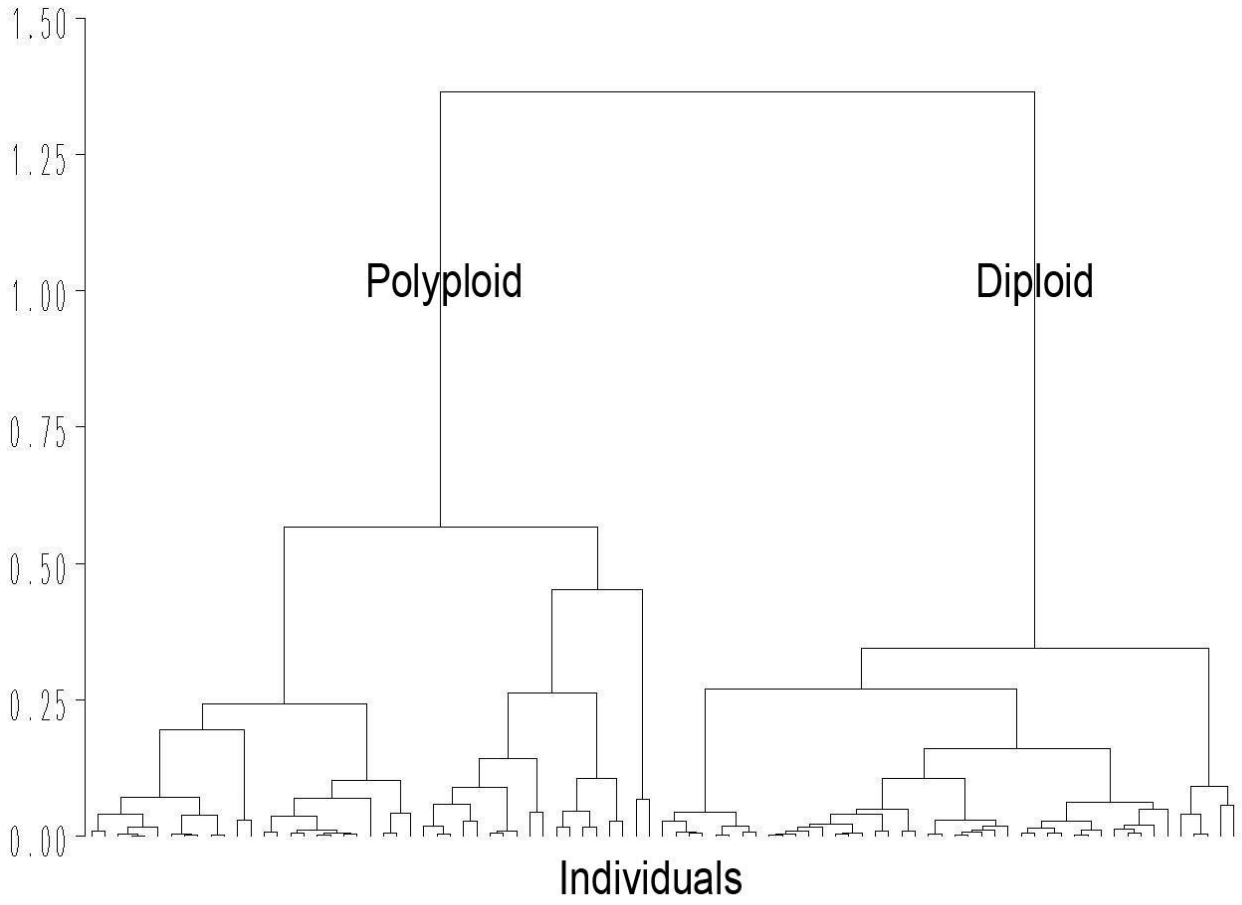


**Fig. 4:** Tree resulting from UPGMA cluster analysis of male erythrocyte circumference. There is a neat clustering of polyploid (3n, 4n) animals in the left clade and diploid animals in the right clade, but no clear separation between 3n and 4n or between LLR and LRR within the polyploid clade.

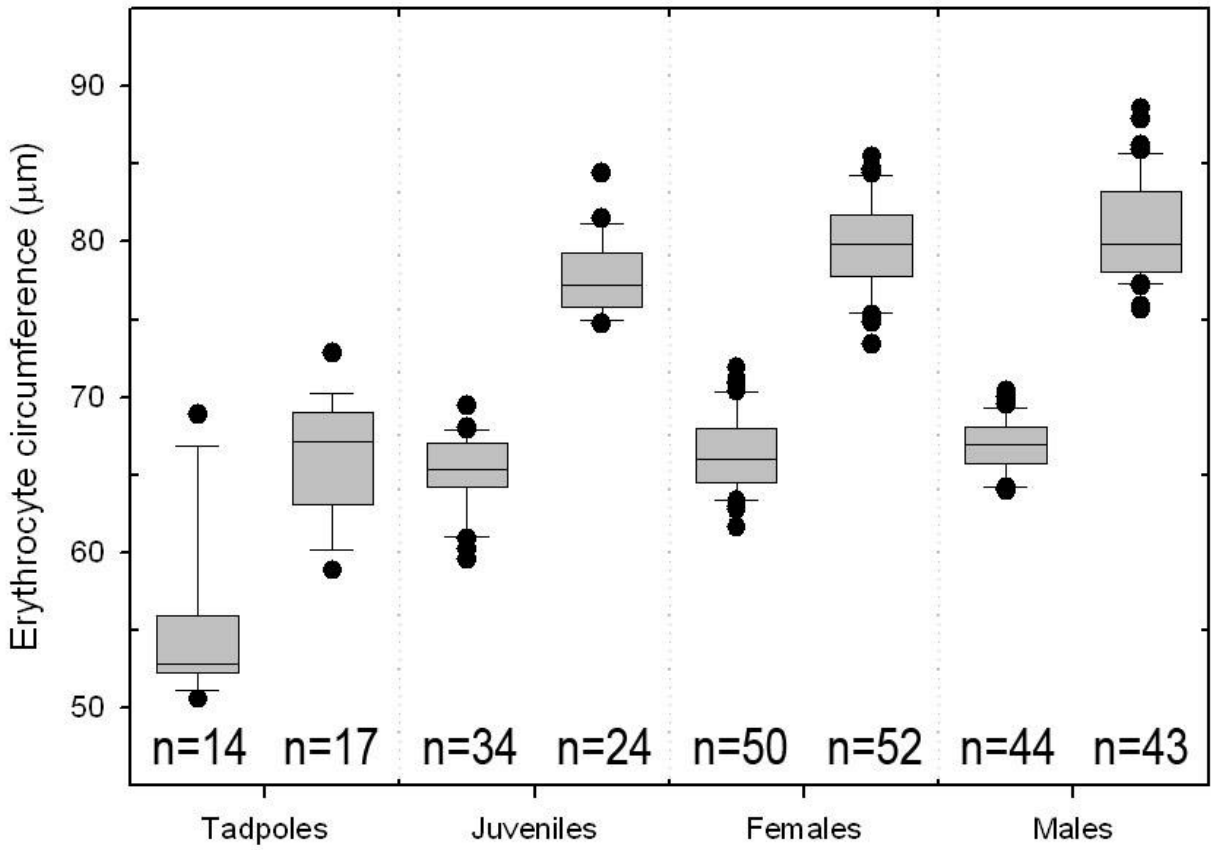


**Fig. 5:** Box plots of RBC circumference for four categories of *R. esculenta*. Diploid values (left) and polyploid values (right) were pooled over all genotypes within each of the four categories. Triploid-like size of 2 diploid LL-tadpoles causes whiskers of tadpole box plots to overlap. Whiskers delimit the upper and lower 95% confidence intervals, boxes are delimited by 25$^{th}$ and 75$^{th}$ percentiles, lines within boxes are medians, black dots are outliers.

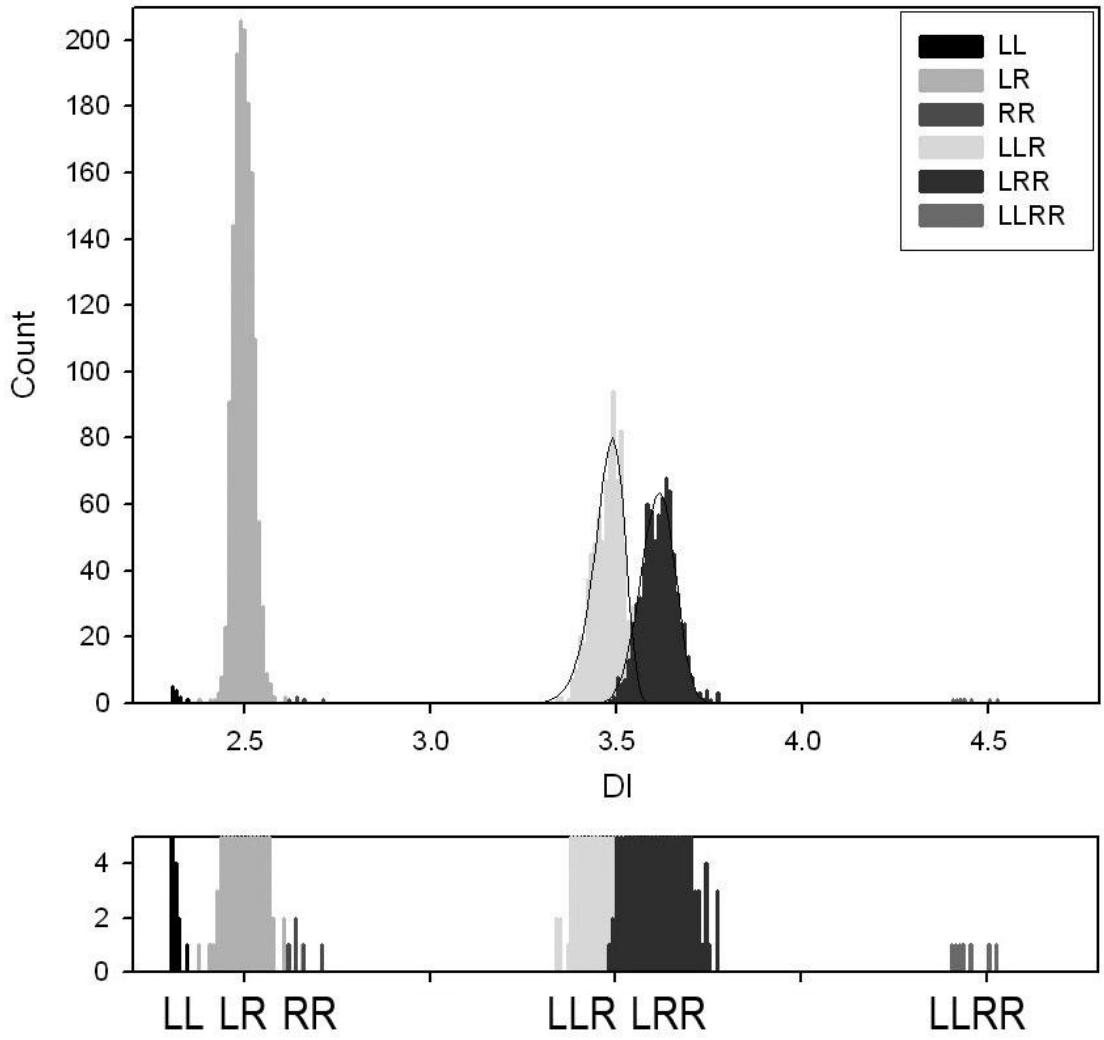


**Fig. 6:** Histograms of RBC DNA Indices (parameter FL2-H (peak fluorescence), category 1 (high quality) results, and years 2002-2004). Distribution curves of LLR and LRR genotypes are Weibull-fitted (4 parameters). Diploid, triploid and tetraploid genotypes are well separated, whereas an overlap between the two triploid types is apparent. Lower part of the figure shows a magnification of low y-values.

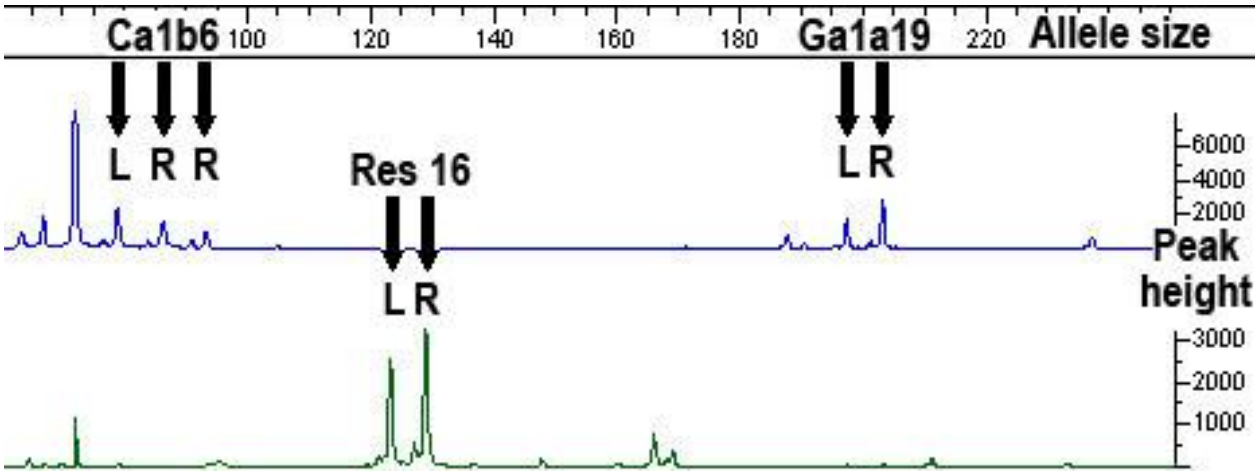


**Fig. 7:** Microsatellite analysis. While the primer Ca1b6 (top lane, left) shows one L- and two R-genome specific allele peaks, Ga1a19 (top lane, right) and Res16 (bottom lane) show only one L- and R-specific allele peak each, but with LRR allele peak height ratios (R-peak is larger than L peak). The influence of allele size on peak ratios is discussed in the text.

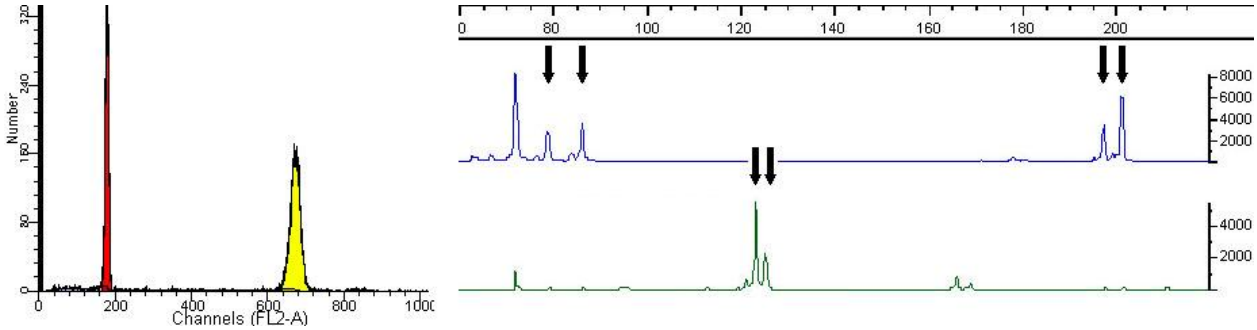


**Fig. 8:** While flow cytometry (left) shows LRR-specific DI values (DI=3.77), microsatellite results are contradictory: while Ca1b6 and Ga1a19 (top lane) also indicate an LRR genotype, Res16 (bottom lane) clearly shows typical LLR allele peak height ratios. CV values of flow cytometry peaks, which are sensitive to aneuploidy, are in normal ranges (2.31% for the standard, left, 1.93 for the sample on the right)

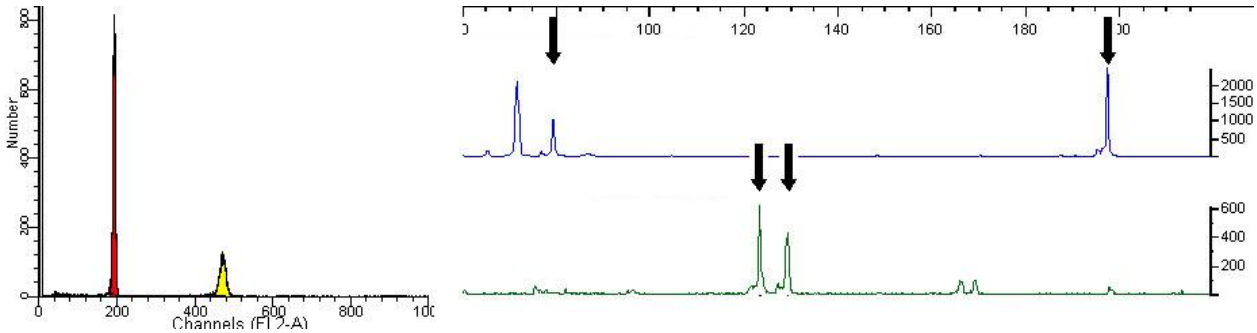


**Fig. 9:** A typical example for findings of a surplus allele. While flow cytometry (DI=2.37) suggests that the adult frog from central Sweden is an LL diploid, this is supported by the microsatellite primers Ca1b6 (top lane, left) and Ga1a19 (top lane, right), showing both only a single L-allele peak. Res16 in the bottom lane shows however an additional R-specific peak at 130bp. The opposite case of missing alleles is also encountered when comparing microsatellite results.

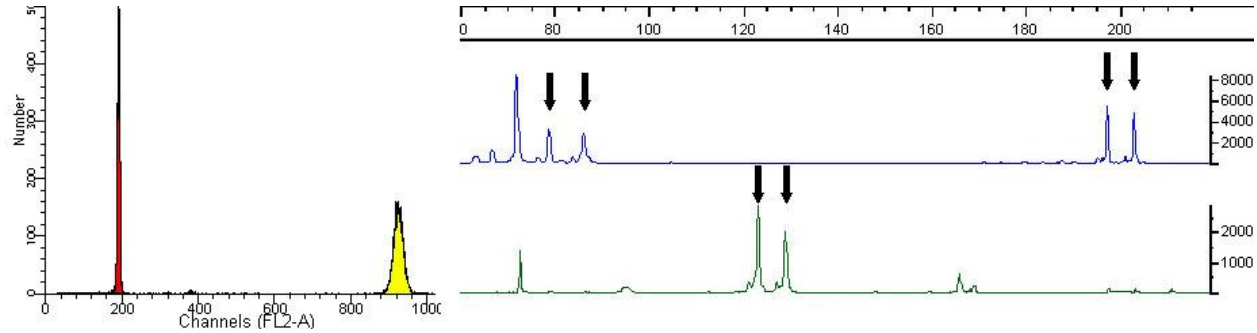


**Fig. 10:** Left: flow cytometry results show that the sampled Swedish frog was tetraploid (LLRR, DI=4.83). Microsatellite analysis (top lane, left: Ca1b6, top lane, right: Ga1a19, bottom lane: Res16) only detects similar peak heights and therefore assigns the animal to LR-genotype.

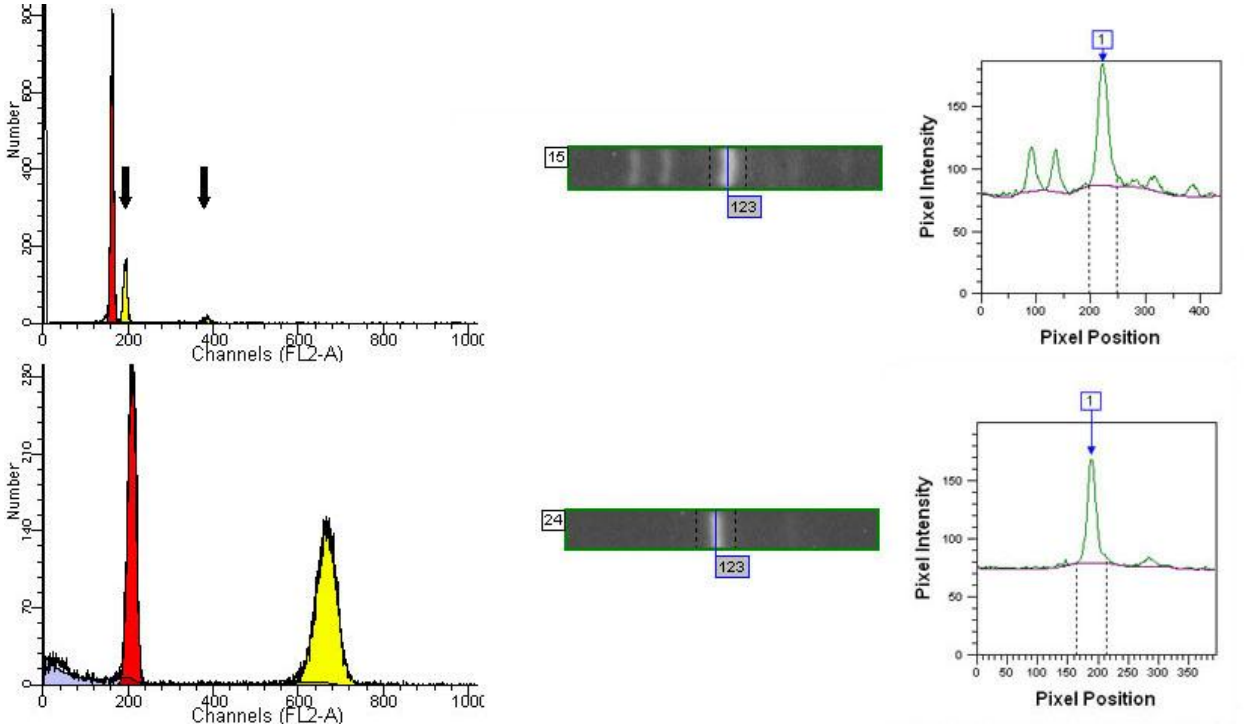


**Fig. 11:** Left: flow cytometry results show that the sampled tadpoles were haploid L (top, DI=1.19; the second arrow shows the G2-peak with DI=2.35) and triploid LLL (bottom, DI=3.29). Microsatellite markers just show a single L-peak (at the example of Ca1b5, allele 123).

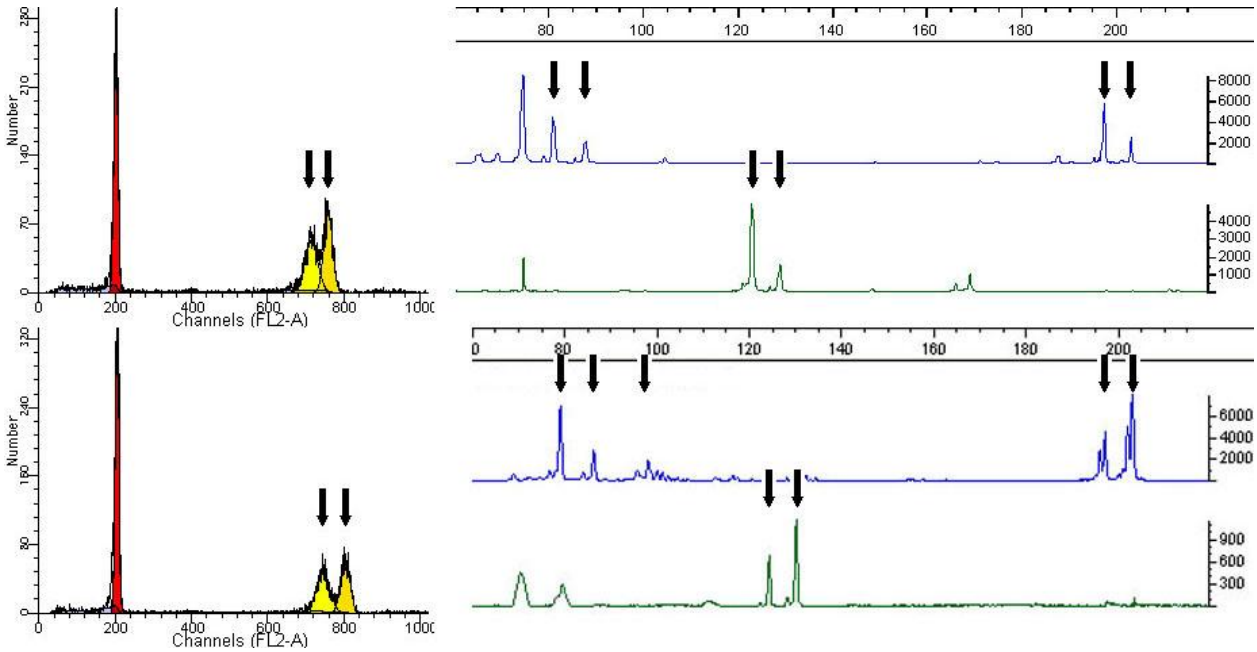


**Fig. 12:** Left: flow cytometry results show that the sampled Swedish frogs were LLR/LRR mosaic frogs. Microsatellite analysis (top lane, left: Ca1b6, top lane, right: Ga1a19, bottom lane: Res16) show either LLR- (top) or LRR-specific peak height ratios (bottom).

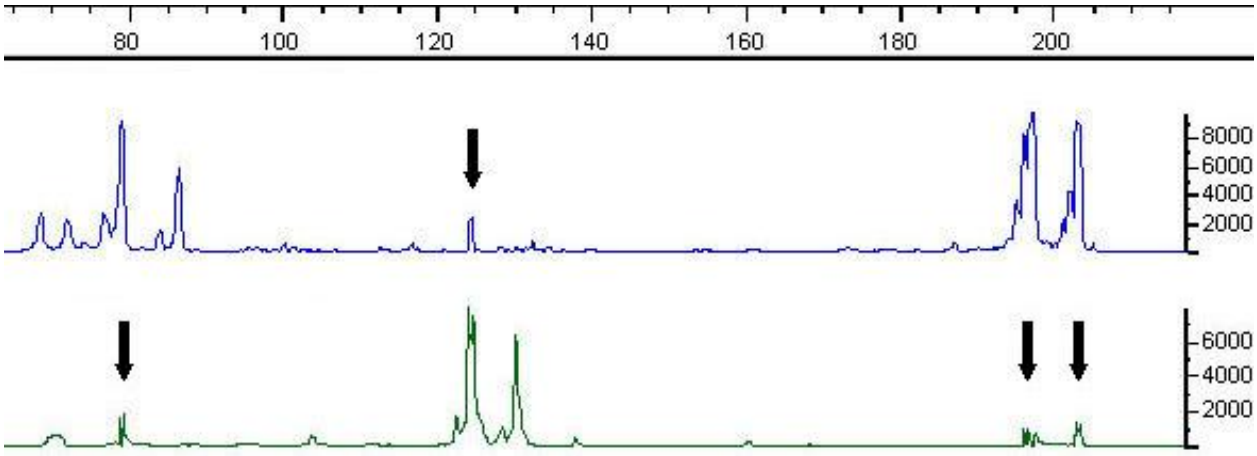


**Fig. 13:** Overload of samples (here at the example of an LLR triploid frog from southern Sweden) leads to cut-off peaks which falsify peak height ratios (here, peak heights should be 2:1), and which also leak into other lanes (arrows show peaks leaking from the green into the blue dye lane and vice versa).

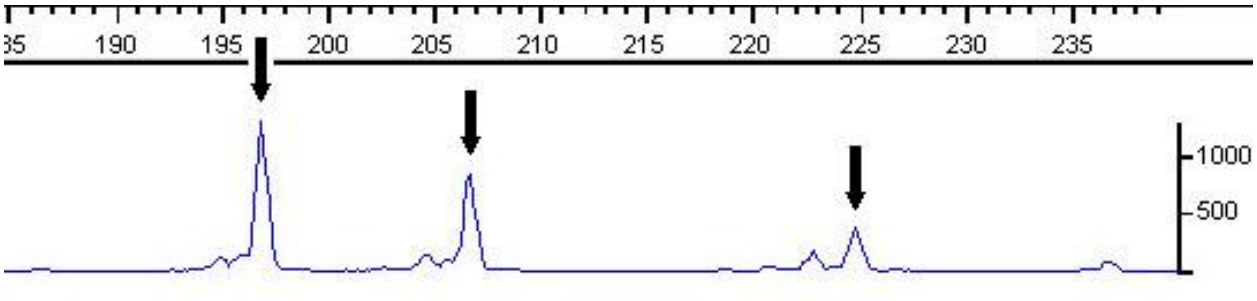


**Fig. 14:** Short allele dominance in the microsatellite primer Ga1a19 at the example of a triploid LRR animal from Świnoujście, Poland. Alleles are at 197bp (L), 207bp (R) and 225bp (R). In theory, all peak heights should be equal (equal gene dosage). Uncorrected, the ratio is 1:0.63:0.31.

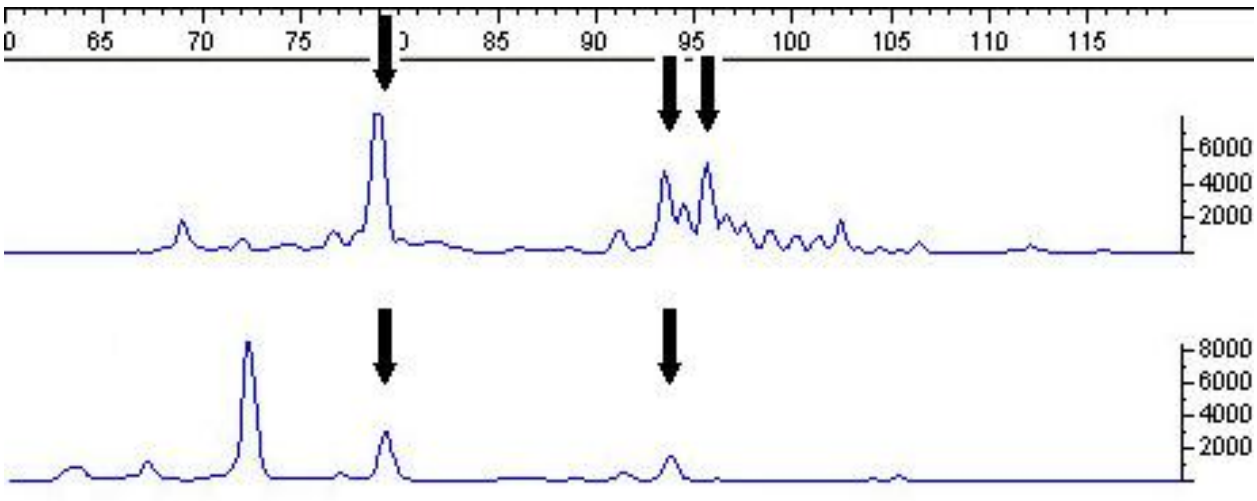


**Fig. 15:** Artefact peaks in the microsatellite primer Ca1b6 encountered in a diploid LR animal from southern Sweden. The first analysis (top) yielded 3 peaks at 79bp (L), and at 93bp and 96bp which lie in the allele range of R genomes. Because of inconsistency with other microsatellite primer results and flow cytometry, a re-analysis was done with the same extracted DNA (bottom), where the second R-peak disappeared. This was a frequent error through several plates, the reason is unknown.

# CHAPTER 2

## Genotypic composition of *Rana esculenta* population systems in Sweden

CHRISTIAN JAKOB, MARTINA ARIOLI & HEINZ-ULRICH REYER

## Abstract

Pure hybrid populations of the water frog *Rana esculenta* are exceptional in many aspects. Besides overcoming the alleged hybrid disadvantages, *R. esculenta* (LR genotype) normally lives in obligate sympatry with one of its parental species, either *R. ridibunda* (RR) or *R. lessonae* (LL), parasitizing it sexually due to its special reproductive mode of hybridogenesis. In pure hybrid populations, triploid hybrid specimens provide the system's stability and viability instead of the parental species. We could show that in Southern Sweden, contrary to assumptions made by previous investigators, the three main genotypes (diploid LR and the two triploid forms LLR and LRR) occur together simultaneously in most of the ponds. Low frequencies of tetraploid and mosaic animals were also unexpectedly present in some ponds, as well as other special genotypes. Genotypic compositions changed significantly in the years 2002-2004. The amount of LR genotypes was steadily increasing, while mainly LLR proportions were decreasing. With the exception of 4 animals, all adult frogs were of a non-parental genotype. Finally, we could show a strongly skewed sex ratio for LRR and RR animals towards females and less pronounced for LLR and LLRR genotypes towards males, providing some support for the theory of L-genome-linked male determining factors.

In the Östergötland district, a newly discovered population could be identified as mixed population of *R. lessonae* and diploid *R. esculenta*. This is the first report of this population type in Sweden.

*Keywords: Rana esculenta*, genotype, diploid, polyploid, pure hybrid system, LE-system, hybridogenesis, Sweden

## Introduction

The European edible frog, *Rana* kl *esculenta* L., is regarded as one of the key examples of how taxa of hybrid origin can defy the often proposed "evolutionary dead-end": Stemming from hybridizations between the pool frog *R. lessonae* Camerano (genotype LL) and the lake frog *R. ridibunda* Pallas (RR), *R. esculenta* (LR) is actually the most widespread water frog taxon in Europe and was shown to form apparently long-term stable populations, mostly in sympatry with one of its parental species (Uzzell 1982). The reason for the common coexistence of *R. esculenta* with either *R. lessonae* or *R. ridibunda* lies in its reproductive mode. The edible frog reproduces hybridogenetically (also known as hemiclonally, see Schultz 1969), a reproductive mode also known from certain fishes, stick insects, and salamanders (Vrijenhoek 1989, Hedges et al. 1992, Mantovani and Scali 1992). By excluding one half of its genome prior to meiosis, *R. esculenta* transmits the other part clonally to its gametes (reviewed in Graf and Polls Pelaz 1989). In the case of mixed populations of *R. lessonae* and *R. esculenta* (a so-called LE-system), the edible frog excludes the L-part of its genome and passes on the R-part clonally. In *ridibunda-esculenta* systems (RE-systems), gamete exclusion works conversely (reviewed in Plötner 2005). Homotypic mating between *R. esculenta* lead to inviable offspring of parental genotype (LL or RR, respectively) because through repeated clonal inheritance, deleterious mutations have accumulated which are then present in a homozygous state (a principle called Mullers ratchet, Muller 1964). In general, *R. esculenta* is therefore obligatory sympatric with one of its parental species, which it then sexually parasitizes.

Besides the common LE- and RE- systems, several exceptions are known, for example pure hybrid systems. In pure hybrid systems, the parental genotypes (LL and RR) are absent among the adults. Instead, triploid LLR and LRR animals, occurring together with diploid LR, take over the role of the parental genotypes by excluding the genome in least copy number and propagating the double-copy genome after normal meiosis (Günther et al. 1979). Such pure hybrid populations are mainly known along the northern distribution range of *R. esculenta* (Günther 1990, Plötner 2005), e.g., in

Northern Germany (Günther and Plötner 1990, Berger and Berger 1994), Poland (Ogielska et al. 2001, Rybacki and Berger 2001), Denmark (Fog 1994, Christiansen et al. 2005), and Sweden (Ebendal 1979).

In Sweden, the focus area of this study, the following water frog population systems have been reported previously:

a. Pure populations of the pool frog, *R. lessonae*, along the Northern Uppland coast, Central Sweden (area 1 in Fig. 1).

b. Pure hybrid populations of the edible frog, *R. esculenta*, in South Western Skåne (Scania), Southern Sweden, some 600 km south of the *R. lessonae* populations (area 2 in Fig. 1).

Additionally, several water frog localities lying between the aforementioned population systems were reported earlier along the Eastern Swedish coast (areas 3-7 in Ebendal 1979).

The isolated occurrence of *R. lessonae* in Central Sweden has sparked the interest of many scientists, resulting in a multitude of publications including investigations of their relationship with other *R. lessonae* populations in Northern Europe (Ebendal and Uzzell 1982, Sjögren Gulve 1991, 1994, Wycherley et al. 2001, Zeisset and Beebee 2001, Tegelström and Sjögren-Gulve 2004, Snell et al. 2005). Therefore, their genotypic composition is well known and undisputed. In this publication we concentrate on the other two localities. After some preliminary investigations in Southern Sweden by (Ebendal and Uzzell 1982), no further systematic studies of these pure hybrid populations were conducted. Sample sizes were generally small and traditional methods used for determination of genotypes (morphology, morphometry, serum electrophoresis) often lack discriminatory powers (Jakob and Arioli, chapter 1 in this publication). Finally, the actual composition of the additional water frog communities in Eastern Skåne and along the Eastern Swedish coast mentioned in (Gislén and Kauri 1959, Ebendal 1979) remains unknown because they have apparently gone extinct, either already before 1979, or until the late 1990s (Kvindall 1998, J. Pröjts, pers. comm.). In the course of an inventory of two presumed *R. esculenta* localities, Jan Pröjts of

Ekologgruppen i Landskrona AB could confirm acoustically the presence of water frogs in Lake Vindommen at Hannäs (Östergötland district), which was detected by locals in 1975 (Söderbäck 1984). In 2004, we discovered another population of water frogs in a pond near Hannäs.

The distribution maps in Günther (1990) and in Plötner (2005) show therefore an inaccurate picture for Swedish water frogs today, because they rely on historical data, whereas Fog et al. (1997) use for their distribution map of *R. esculenta* only the confirmed localities mentioned in Ebendal (1979), without the East Skåne populations.

This study is the first large-scale investigation on the composition of pure hybrid populations of the *R. esculenta* water frog complex (EE), and also reports the first finding of a third water frog population system in Sweden, the LE-system.

## Methods

### *Skåne samples*

The sampling was conducted in the years 2002-2004 in an area located in South-Western Skåne (Scania), Southern Sweden (area 2 in Fig. 1).

Prior to and during the first year, we have investigated a total of approximately 140 ponds in the region, finally selecting 23 of them for detailed analysis. Selection was based on criteria such as accessibility of the pond, number of frogs present, as well as the practicability of catching frogs in the pond (depth, riparian morphology) and the possibility to allow a representative sampling of the population (pond size). Within the 23 ponds, a subsample of 12 ponds ("core ponds") was also surveyed in 2003 and 2004. The ponds were sampled at least twice in the season (May to July) at variable time intervals. Including a total of 514 recaptures, we caught 973 frogs in 2002, (mean n per pond: 42 ± 12), 1180 in 2003 (98 ± 33) and 1080 in 2004 (90 ± 18). In 2003, one newly dug pond was sampled once in the season in addition to the 12 core ponds (n=37). In 2004, 9 additional locations along the edge of the *R. esculenta* distribution in Southern

Sweden were sampled once in June or July (283 frogs, mean n per pond: 31 ± 3). For a list of the sample sites, see Table 1.

In 2002, only seemingly sexually mature frogs were collected, i.e., frogs larger than about 45mm (snout-vent length), whereas in the following years, also subadult frogs entered the sample.

### *Östergötland samples*

In June 2004, the pond Lindalsgöl near Hannäs in Östergötland (area 3 in Fig. 1) was sampled once (40 frogs).

### *Sampling procedures*

Frogs were caught by hand during night time with the help of a flashlight and transported to the Stensoffa field station of the University of Lund. Within 24 hours, the frogs were measured, weighed, and individually marked with a RFID PIT tag (Trovan ID101, Trovan Ltd., UK), except for animals from populations that were sampled only once and subadults smaller than 30mm. One phalanx of the fourth toe was clipped for DNA analysis and stored in Ethanol at -20°C until analysis. Additionally, about 30-50µl blood was taken from a web vein for flow cytometric analysis with a heparinized capillary tube (70µl Micro-Hematocrit Capillary Tubes, VWR International, West Chester USA) and stored in citrate buffer (D-(+)-glucose 475 mM, Sigma G8270; trisodium citrate 40 mM, Sigma-Aldrich S4641; dimethyl sulfoxide 5%, Sigma D8418; pH 7.6) at -80°C until analysis. Within 24 hours, frogs were released at their capture sites.

### *Genotype determination*

Genotype determination followed the procedures described by Jakob and Arioli (chapter 1 in this publication) by means of flow cytometry of nucleated red blood cells, and by gene dosage effects in the microsatellite primers Ca1b5 (Garner et al. 2000), Ca1b6, Ga1a19 (Arioli 2007) and Res16 (Zeisset et al. 2000). Samples yielding contradictory results were re-analyzed and, if contradictory results were persistent, referred to as "Mixed2n" for diploid and "Mixed3n" for triploid animals. Samples that could not be assigned to a genotype because of insufficient sample quality (tissue and/or blood) were left out of the analysis. Specimen with single missing microsatellite

primer alleles were assigned to a genotype according to the other microsatellite primers and flow cytometric results, but were recorded specifically. Mosaic animals (with differing DNA contents in different cells) were detectable by flow cytometry only, showing 2 distinct peaks of luminescence (see Jakob and Arioli, chapter 1 in this publication). By the same method it is also possible to determine aneuploidy with an incomplete set of chromosomes.

*Statistical analyses*

To test for significant changes in global genotype composition between the years, we have performed ANOVAs pooled over all samples from all ponds, grouped by males, females, adults and juveniles for the three main genotypes LR, LLR and LRR. To test for pond and year effects on genotype frequency, we performed an ANOVA on the sub-sample of the 12 core ponds (which were sampled over all three years). To test for possible systematic temporal shifts in genotype composition within a year (e.g., some genotypes appearing and leaving earlier in the breeding season compared to others), an ANOVA with relative genotype frequency classified by sampling month and controlled for year effects was performed for males, females, juveniles and adults pooled over the core ponds.

Statistical analyses were performed with SAS 9.1.3 SP3 for Windows (© 2002-2003 SAS Institute Inc., Cary, NC, USA.), percentage data was arcsine squareroot-transformed before statistical analysis ($x' = \arcsin\sqrt{x}$) to assure a nearly-normal distribution. Graphs were produced using SigmaPlot 2002 v8.02 for Windows (© 1986-2001 SPSS Inc., Chicago, IL, USA).

## Results

The main adult genotypes found in Southern Sweden were diploid LR, together with triploid LLR and LRR. Occasionally, also tetraploid adults were found, as well as specimen classified as “mixed” because results from genotype determination methods were consistently contradictory. With the exception of pond 089 in 2003 and pond 154

in 2004, where four individuals of the parental RR genotype were found, parental genotypes (LL or RR) were completely absent from adult samples, although they were occasionally found among juveniles. When looking at combined samples in 2002, 22 out of 23 sampled ponds had an excess in triploid animals (LLR and LRR genotypes combined, Fig. 2). The amount of diploid adult LR animals varied between 7.7% in pond 001 and 53.1% in pond 032 (mean: 28.9%, stdev: 11.6%). Two ponds (123 and 137) consisted only of LR and LLR animals, in a further pond (138), only LR and LRR animals were found. In all other ponds, all three main genotypes were present at the same time at varying rates. The fact that the three main genotypes were present in the majority of the sampled ponds at the same time was true also for 2003, (except for pond 001: no LRR, see Fig. 3), and for 2004 (except for pond 154: no LLR; and pond 161: no LRR; see Fig. 4). Relative genotype frequencies within a pond can change quite dramatically within and between years (as can be seen in Figs. 5, 6, 7, and 8, shown for our 12 core ponds). Also, overall genotype frequencies changed over the years (see Fig. 9): When performing an ANOVA (PROC GLM in SAS) over all pooled samples considering only LR, LLR and LRR genotypes, there was a significant year-effect on the percentage of caught LR genotypes (and consequently, the relative amount of diploid animals in the samples) between the years (2002-2004) for adult frogs ($p<0.0001$, $F=11.60$, $df=2$), female frogs ($p<0.01$, $F=8.41$, $df=2$), male frogs ($p<0.05$, $F=4.34$, $df=2$), and juvenile frogs (only 2003 and 2004 samples; $p<0.05$, $F=6.95$, $df=1$). Post-hoc Scheffé's tests showed that significant changes in relative LR, respectively diploid frequencies occurred between 2002 and 2004, and between 2003 and 2004. The relative increase of LR animals was mostly at the cost of LLR genotypes. When performing the same analysis for them, there were significant differences for adults ($p<0.0001$, $F=13.68$, $df=2$), females ($p<0.05$, $F=8.85$, $df=2$), males ($p<0.0001$, $F=13.43$, $df=2$), but not for juveniles ($p>0.05$, $F=0.47$, $df=1$). LRR frequencies in the pooled sample did not significantly change over the years, however (data not shown), except for juvenile frogs ($p<0.05$, $F=4.32$, $df=1$).

While adult LR and mixed genotypes consisted about equally of males and females, the sex ratios of the other genotypes were skewed (shown in Fig. 10). LRR, RR and Mosaic

animals exhibited a strong female bias, whereas LLR and LLRR animals were, to a lesser extent, biased towards males.

When comparing the 12 core ponds which were sampled every year by means of an ANOVA (PROC GLM in SAS), performed for each genotype, significant effects of pond, year, and also interaction effects between both were found for some groups (see Table 2). There were genotype frequency changes within each year between sampling events. But when testing each pond for year and sampling month effects, there was no systematic effect of sampling month on genotype frequency, except for pond 014, where a significantly higher frequency of LR adult frogs was caught in May than in June ($p<0.05$, $F=23.40$, $df=1$), and for LR females in pond 032 which were caught with lowest frequency in June, highest in July and medium frequency in May ($p<0.05$, $F=3.68$, $df=2$). When testing over all ponds, genotype frequencies were not affected by sampling month.

In contrast to the pure hybrid, diploid-polyploid population system in Skåne, the population in Östergötland is an LE-system (Fig. 11), consisting of only diploid *R. esculenta* (LR), together with diploid *R. lessonae* (LL). This population system is found also in large parts of Central Europe.

## Discussion

### *Skåne*

We could show that *R. esculenta* forms pure hybrid populations in Skåne, as was suggested in Ebendal (1979) and Ebendal and Uzzell (1982). Because of the low sample sizes analyzed in these publications, the authors had to include the possibility for the presence of adult genotypes in low frequency. With our analysis of nearly 3000 individual specimen, we can rule this possibility out. Parental genotypes are formed by non-assortative mating, but their frequency in the different life stages is gradually decreasing (Arioli and Jakob, chapter 6 in this publication), until they are no longer present in the adult samples. There were 4 exceptions in our data set: 1 female from pond 089 and 3 females from pond 154, which all exhibited RR genotypes. While the

animal from 089 was most probably a juvenile animal that was misclassified by our arbitrary size limit, the animals from 154 were definitely adults. The concentrated presence of these RR genotypes in this pond (situated on a golf course) is puzzling and should be monitored. Although introduction of RR animals cannot be ruled out completely, it is nevertheless improbable judging from microsatellite and mtDNA data (Arioli 2007).

In the past, the (now extinct) populations in South Eastern Skåne have been described either as "morphologically slightly similar" to RR (Ebendal 1979), or have been found to be of the LR and LRR genotype (Ebendal and Uzzell 1982). The specimens from South Western Skåne, however, have always been described as LR and LLR genotypes. We could show that in fact all three main genotypes (LR, LLR, and LRR) occur in most of the ponds simultaneously in varying proportions. Contrary to the simple and reportedly most widespread situation of LR-LLR populations modelled by Som and Reyer (2006), LRR animals are quite common and, as shown by Jakob and Arioli (chapter 5 in this publication) and Arioli and Jakob (chapter 6 in this publication), are viable. That the three main genotypes may occur together in the same pond was also shown by Christiansen et al. (2005) for some pure hybrid populations in Denmark, although one triploid genotype was always dominant over the other. In contrast to this publication, however, we have found also adult tetraploid animals in our sample, thanks to the incorporation of flow cytometric analyses for ploidy determination in our study. Tetraploid animals may provide a step towards the formation of a new, independent species by reintroducing normal meiosis. In the diploid/polyploid hybrid *Squalius alburnoides* system, tetraploid animals are regarded as a possibility for a return to normal sexual reproduction, although they are in low frequency (Pala and Coelho 2005). Although Vrijenhoek (2006) has recently emphasized the importance of tetraploids in speciation processes, the role of tetraploid animals in water frogs remains to be investigated.

The "mixed" animals (where genotype determination methods yielded contradictory results) seem to indicate the introgression of L- genes or genome parts into the R

genome and vice versa (see Jakob and Arioli, chapter 1 in this publication). Although they occur at low frequencies, they are widespread across the sampled ponds. The detection probability of such mixed genotypes will further increase with the number of microsatellite loci used for genetic investigations.

"Mosaic" animals were detectable only by flow cytometry (see Jakob and Arioli, chapter 1 in this publication). They exhibit red blood cells with varying genotypic content, e.g., LLR and LRR, LLL and LLR, LL and LR. Such animals were found only rarely among adult frogs and seem to only exceptionally survive.

There were no aneuploid animals detected in our samples of juvenile and adult frogs. Aneuploid samples should be detectable with flow cytometric analysis, showing unusual broad fluorescence peaks (resulting in high coefficients of variation) or unusual relative DNA indices compared to standard cells (Tiersch and Wachtel 1993, Lowcock et al. 1997, Sharbel et al. 1997, Bihari et al. 2003).

Although rarely found, the exceptional genotypes and unusual genomic combinations demonstrate the complexity of this reproductive system and the inherent possibility for new evolutionary pathways in pure hybridogenetic water frog populations.

There was a notable sex bias for genotypes with an excess of R-genomes (LRR, RR) to be female. This supports the theory that the male determining factors in hybrids of *R. lessonae* and *R. ridibunda* are linked to the L-genome (see Table 3). The low-frequency presence of LRR-males in some of the ponds, however, cannot be easily explained by this presumption and has to be investigated further.

The population stability was low; there was a general significant increase of the relative amount of LR-animals in Skåne over the years at the expense of LLR-animals. This trend occurred for both males and females and could be observed to continue in 2005 (D. G. Christiansen, pers. comm.). If this change in relative frequencies is due to natural fluctuations in pure hybrid populations or due to an extrinsic factor remains to be investigated with long-term studies. Due to the fact that offspring ploidy is directly determined by the parent's ploidy (triploid animals and diploid males normally produce haploid gametes, diploid females produce haploid and diploid gametes), a high

percentage of triploid parents leads to a high percentage of diploid offspring, whereas a high proportion of diploid females enhance the frequency of triploid offspring. This may indicate that, in fact, transitions in relative genotype frequencies are a natural phenomenon in these populations. A study on Danish pure hybrid populations concluded that those were stable over time (Christiansen et al. 2005). The sampling regime, sample sizes, compared time spans and partly also the genotype determination methods differed greatly from our study, however, so that further analyses may come to similar results.

### *Östergötland*

The detection of an LE- water frog system in Sweden was very surprising. Although this population lies very well in the water frog distribution range still reported in the early to mid-20$^{th}$ century in Southern Sweden (Gislén and Kauri 1959, Ebendal 1979), this particular population was only known to locals (Söderbäck 1984) who described the frogs simply as "edible frogs". The genotypic composition of other historically reported populations along the eastern coast of Southern Sweden remains unknown, because these populations have never been investigated on their genotypic composition (Ebendal 1979) and most probably have gone extinct until the end of the last century (Kvindall 1998, J. Pröjts in litt.). This LE-population may be considered as an intermediate link between the LL-system in Uppland and the EE-system in Skåne and could be the remnant of colonization after the last ice age. We know from Southern Sweden that selection acts against the parental forms LL and RR. In the Baltic States, at latitudes comparable to Östergötland, the LE-system is the most widespread, showing the adaptation of this population system to such latitudes. It may well be that *R. esculenta* is at a disadvantage at higher latitudes, but there are no field or experimental data of differential larval or adult performance of different genotypes from either water frog populations north of Skåne. Further insight into the status of the Östergötland population (native vs. introduced) could be gained by genetic investigations; a first analysis with few genetic markers did deliver some support for its native status (Arioli 2007). In any case, this is the first discovery of a mixed *lessonae-esculenta* population system in Sweden. Besides the pond Lindalsgöl and nearby Vindommen Lake, no further

localities of green frogs in the region have been found yet, so the area should be subjected to protective measures.

## Acknowledgements

We would like to thank Sandra Röthlisberger for help with the lab work. The group of Rolf Zinkernagel at University Hospital Zürich provided us access to their FACSCalibur flow cytometer. Bo Baldetorp of Lund University Hospital and Eva Niederer of the Institute for Biomedical Engineering IBT at the ETH Zürich provided practical and technical advices for flow cytometric analysis. At several occasions, we had guest researchers helping us with frog catching, namely Ditte G. Christiansen, Jon Loman, Rachel Muheim, Michael Tobler, Trent J. Garner, and Stefan Goedderz.

The work was funded by the Swiss National Science Foundation, grant No. 3100-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-18591-02, 522-9286-03, 522-6571-04) and by Länsstyrelsen Östergötland (522-1923-04).

## Author contributions

CJ and MA contributed equally to this work. Both authors carried out all field- and lab work together. CJ performed statistical analyses and wrote the paper. CJ and MA authors discussed the results and MA and HUR commented on the manuscript.

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## Tables

**Table 1:** Ponds sampled in 2002-2004.

| | | | Number of sampling events | | |
|---|---|---|---|---|---|
| Pond | Region | Coordinates | 2002 | 2003 | 2004 |
| 001 | Skåne | 55°35'17"N 13°21'15"E | 2 | 2 | 2 |
| 010 | Skåne | 55°34'12"N 13°19'37"E | 2 | - | - |
| 011 | Skåne | 55°34'06"N 13°19'47"E | 2 | 2 | 2 |
| 012 | Skåne | 55°34'09"N 13°19'38"E | 2 | - | - |
| 014 | Skåne | 55°34'08"N 13°19'01"E | 2 | 2 | 2 |
| 021 | Skåne | 55°34'09"N 13°16'42"E | 2 | - | - |
| 023 | Skåne | 55°34'23"N 13°16'55"E | 2 | - | - |
| 024 | Skåne | 55°34'27"N 13°16'49"E | 2 | - | - |
| 032 | Skåne | 55°34'03"N 13°12'53"E | 2 | 2 | 2 |
| 032A | Skåne | 55°34'27"N 13°13'03"E | 2 | 3 | 2 |
| 050 | Skåne | 55°29'33"N 13°08'02"E | - | - | 1 |
| 089 | Skåne | 55°36'34"N 13°23'19"E | 3 | 3 | 2 |
| 101 | Skåne | 55°32'51"N 13°17'04"E | 2 | - | - |
| 102 | Skåne | 55°32'51"N 13°17'13"E | 2 | 2 | 2 |
| 108 | Skåne | 55°33'09"N 13°16'08"E | 2 | 2 | 2 |
| 108A | Skåne | 55°33'11"N 13°16'09"E | 2 | - | - |
| 111 | Skåne | 55°32'06"N 13°12'33"E | 2 | 2 | 2 |
| 112 | Skåne | 55°32'05"N 13°12'44"E | 2 | - | - |
| 123 | Skåne | 55°35'17"N 13°21'07"E | 2 | - | - |
| 126 | Skåne | 55°33'59"N 13°14'12"E | 2 | 2 | 2 |
| 134 | Skåne | 55°33'03"N 13°21'22"E | 2 | 2 | 2 |
| 135 | Skåne | 55°33'12"N 13°21'39"E | 2 | - | - |
| 137 | Skåne | 55°39'14"N 13°24'32"E | 2 | - | - |
| 138 | Skåne | 55°31'32"N 12°55'45"E | 2 | 2 | 2 |
| 139 | Skåne | 55°34'06"N 13°05'35"E | - | 1 | - |
| 142 | Skåne | 55°35'08"N 13°06'42"E | - | - | 1 |
| 147 | Skåne | 55°31'12"N 13°06'18"E | - | - | 1 |
| 151 | Skåne | 55°27'03"N 13°10'17"E | - | - | 1 |
| 154 | Skåne | 55°22'24"N 13°05'32"E | - | - | 1 |
| 155 | Skåne | 55°22'08"N 13°26'14"E | - | - | 1 |
| 159 | Skåne | 55°22'59"N 13°27'01"E | - | - | 1 |
| 160 | Skåne | 55°40'01"N 13°25'48"E | - | - | 1 |
| 161 | Skåne | 55°36'40"N 13°26'18"E | - | - | 1 |
| 401 | Östergötland | 58°06'57"N 16°24'15"E | - | - | 1 |

**Table 2:** Results for an ANOVA (PROC GLM of SAS) with the effects of pond, year, and the interaction pond*year on the main genotypes, by adults, females, males and juveniles. P values <0.05 are printed in bold. Number of samplings in 2002: 25, in 2003: 26, in 2004: 24 (see also Table 1).

| | | | LR | | LLR | | LRR | |
|---|---|---|---|---|---|---|---|---|
| Sex | Effect | df | p | F | p | F | p | F |
| Adults | Pond | 11 | **<0.0001** | 5.61 | **<0.0001** | 22.48 | **<0.0001** | 27.59 |
| | Year | 2 | **<0.0001** | 17.42 | >0.1 | 1.94 | **<0.01** | 6.96 |
| | Interaction | 22 | **<0.05** | 2.16 | **<0.05** | 2.26 | >0.1 | 1.53 |
| Females | Pond | 11 | **<0.0001** | 7.78 | **<0.0001** | 10.99 | **<0.0001** | 14.36 |
| | Year | 2 | **<0.0001** | 25.02 | >0.1 | 0.45 | **<0.01** | 6.60 |
| | Interaction | 22 | **<0.01** | 3.59 | >0.1 | 1.14 | >0.05 | 1.77 |
| Males | Pond | 11 | **<0.05** | 2.49 | **<0.0001** | 9.25 | **<0.0001** | 7.09 |
| | Year | 2 | >0.1 | 1.99 | >0.05 | 2.56 | >0.1 | 0.22 |
| | Interaction | 22 | >0.05 | 1.79 | **<0.05** | 2.00 | >0.1 | 0.43 |
| Juveniles | Pond | 11 | >0.1 | 0.61 | >0.1 | 1.49 | **<0.01** | 4.66 |
| | Year | 1 | >0.05 | 3.96 | >0.1 | 0.17 | >0.1 | 1.96 |
| | Interaction | 11 | >0.1 | 1.06 | >0.1 | 1.42 | >0.1 | 1.07 |

**Table 3:** Schematic table showing the offspring genotypes stemming from possible crosses in pure water frog populations in Southern Sweden (adapted from Arioli 2007), under the assumption that primary hybridizations occurred between LL males and RR females. Subscript indicate the sex of the offspring (m=male, f=female) or whether a gamete carries a female or male determining factor. Offspring types on grey background usually are inviable and die before reaching the adult stage.

| Females | Males Gametes | LLR $L_{f,m}$ | LR $R_f$ | LRR $R_f$ |
|---|---|---|---|---|
| LLR | $L_{f,(m?)}$ | $LL_{f,m}$ | $LR_{f,(m?)}$ | $LR_{f,(m?)}$ |
| LR | $L_fR_f$ | $LLR_{f,m}$ | $LRR_f$ | $LRR_f$ |
| | $R_f$ | $LR_{f,m}$ | $RR_f$ | $RR_f$ |
| LRR | $R_f$ | $LR_{f,m}$ | $RR_f$ | $RR_f$ |

## Figures

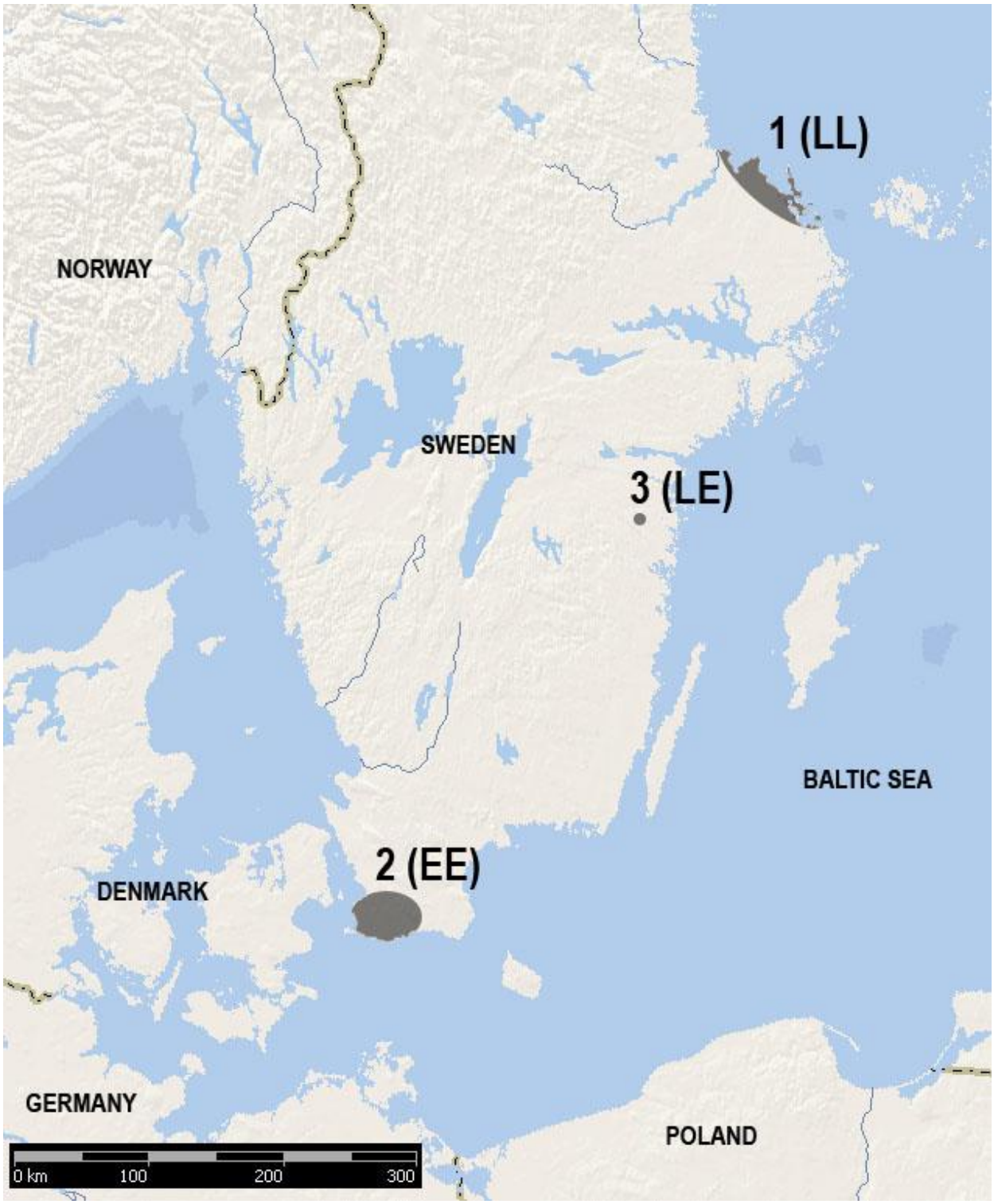


**Fig. 1:** Map of Southern Sweden with the location of pure populations of *R. lessonae* (LL) in Uppland (1), pure hybrid water frog populations (EE) in South-Western Skåne (2), and a recently discovered water frog population in Östergötland (LE, 3). Map: Adapted from Microsoft Encarta 2000 (© 1993-1999 Microsoft Corp., Redmond, WA, USA).

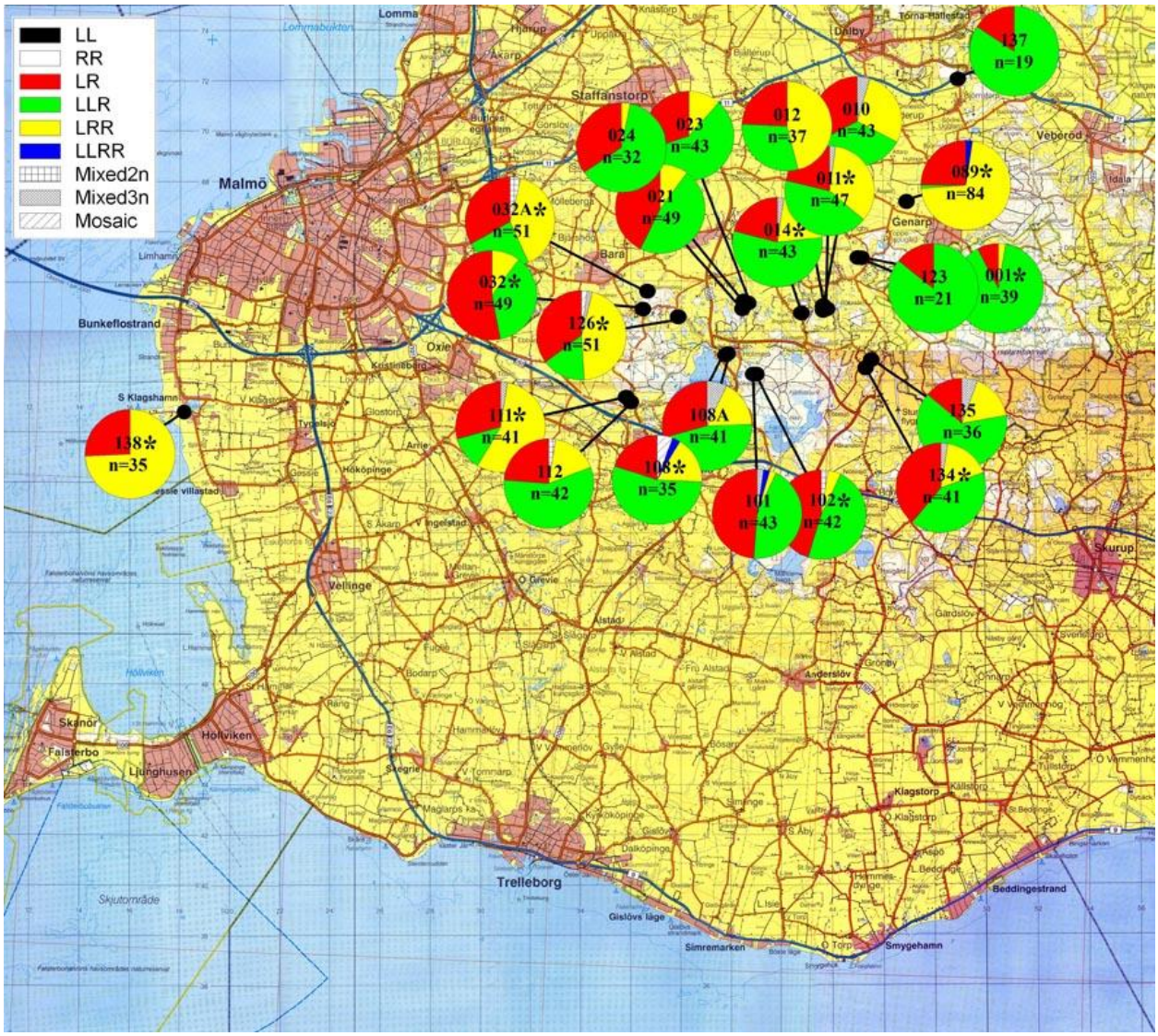


**Fig. 2:** Genotype composition 2002 (adult frogs, pooled over all samplings). "Core ponds" are marked with an asterisk (*). Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

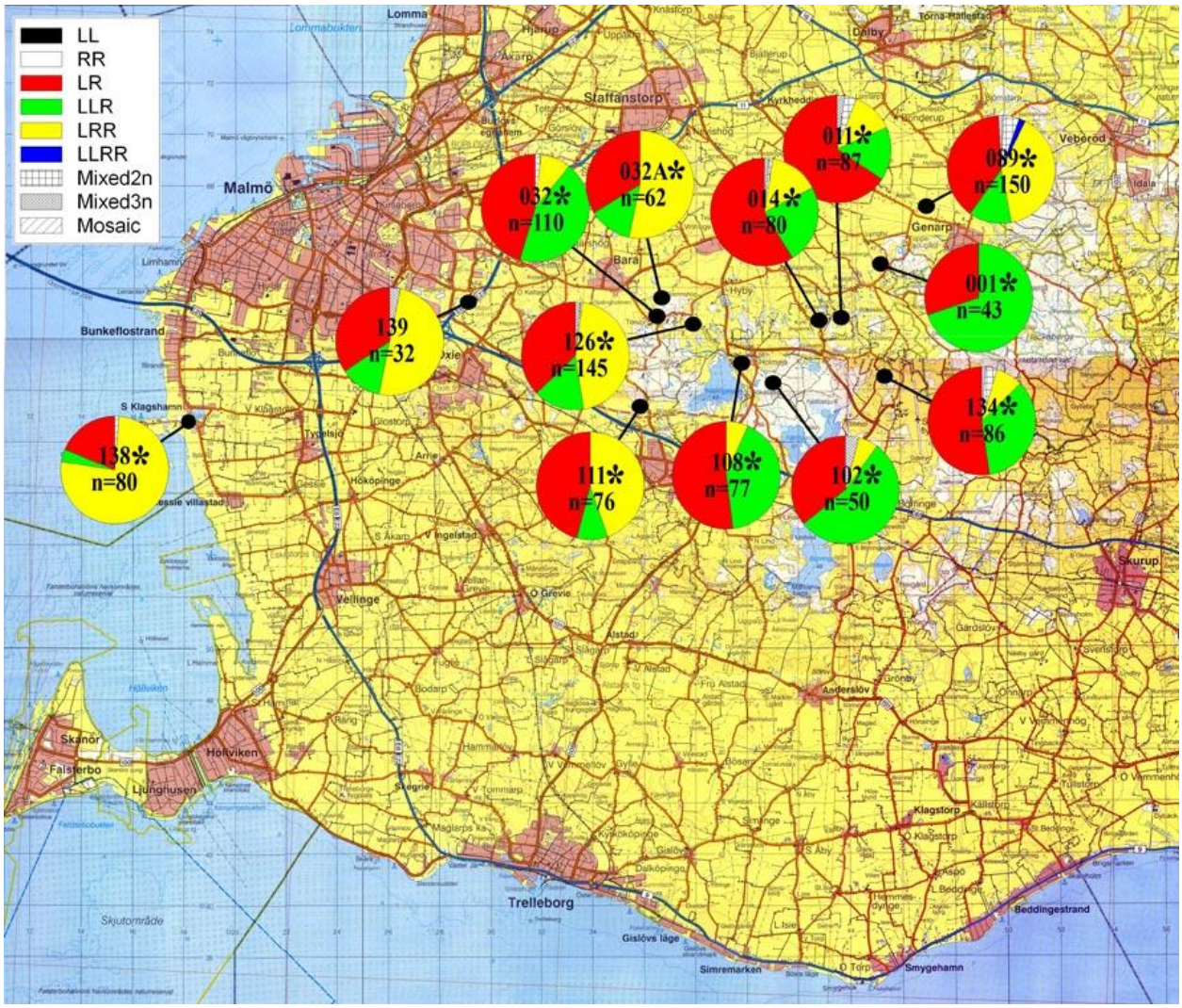


**Fig. 3:** Genotype composition 2003 (adult frogs, pooled over all samplings). "Core ponds" are marked with an asterisk (*). Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

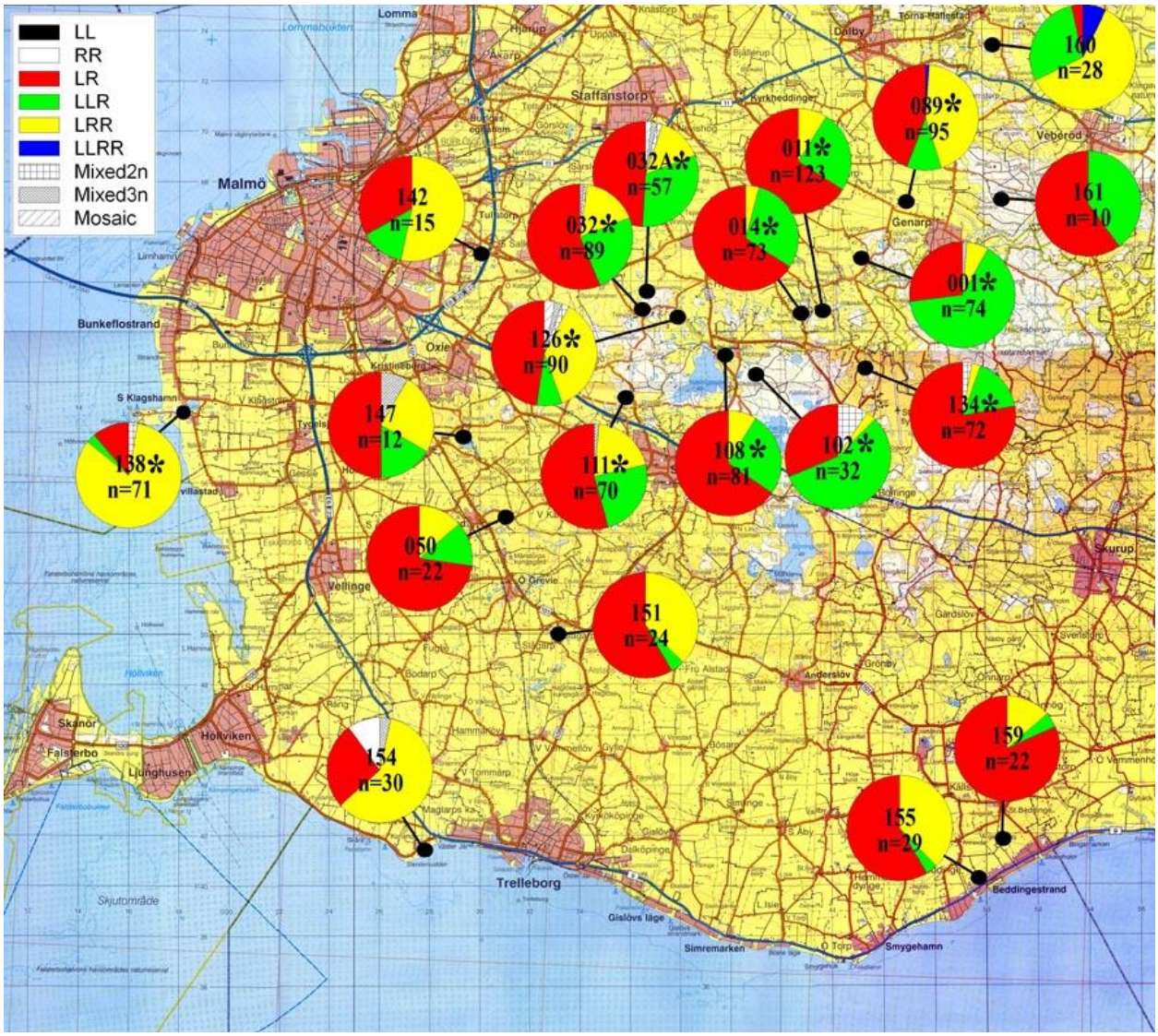


**Fig. 4:** Genotype composition 2004 (adult frogs, pooled over all samplings). "Core ponds" are marked with an asterisk (*). Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

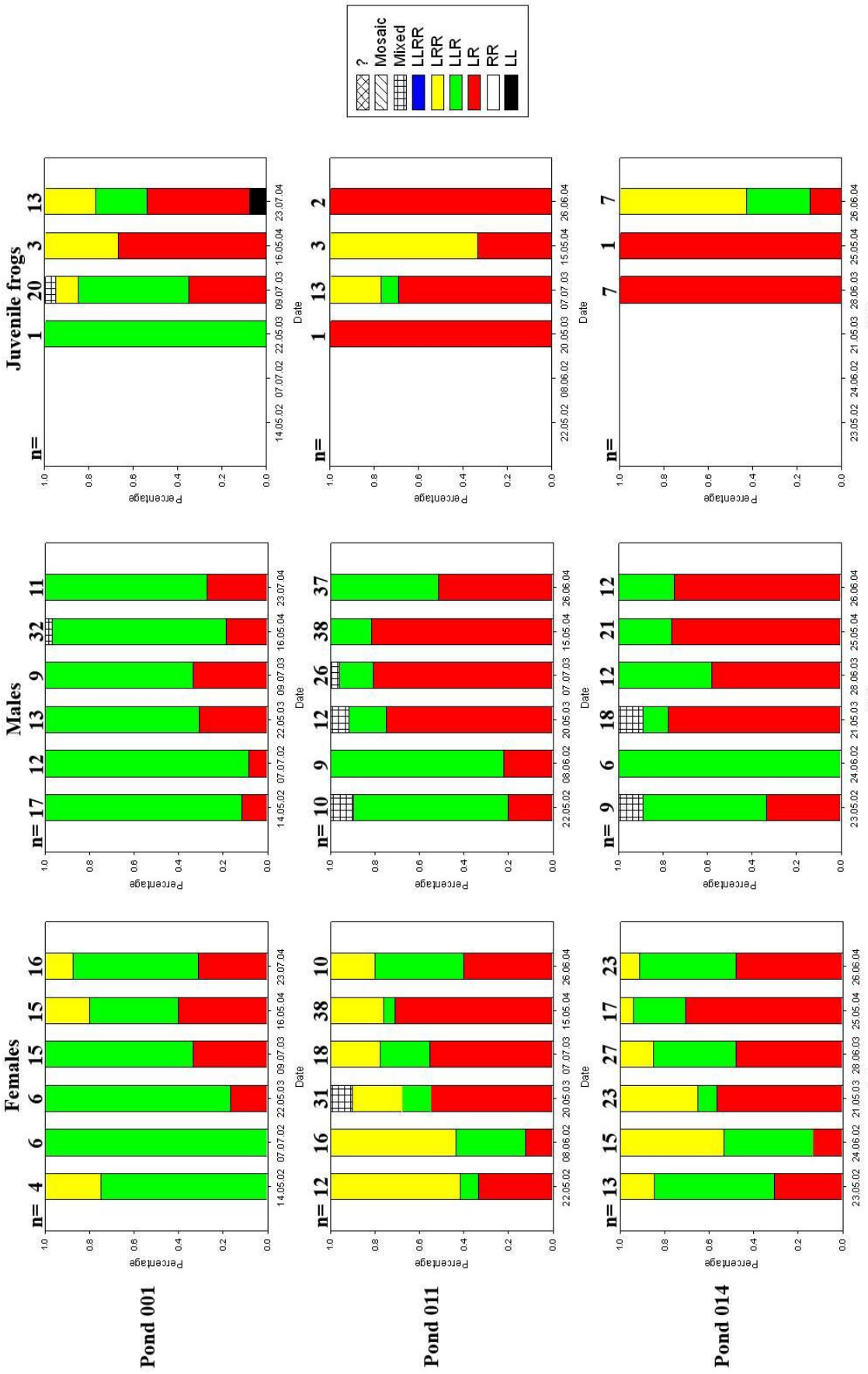


**Fig. 5:** Genotype composition of core ponds 001, 011 and 014, listed for females, males, and juveniles. Sampling occurred from 2002-2004 twice a year; juveniles were not included in every sampling.

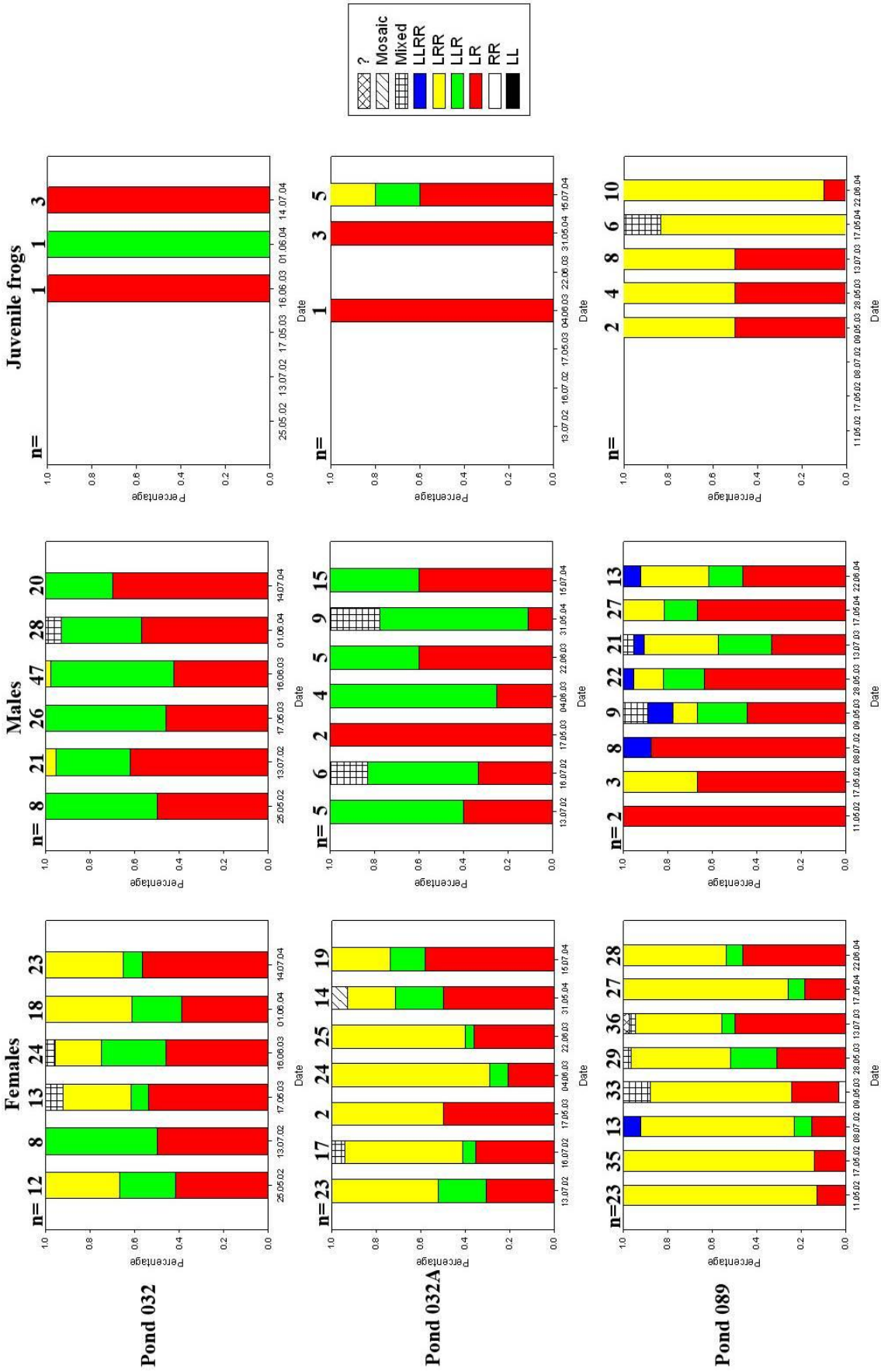


**Fig. 6:** Genotype composition of core ponds 032, 032A and 089, listed for females, males, and juveniles. Sampling frequency: 2 (032, 032A) or 3 (089) in 2002, 2 (032) or 3 (032A, 089) in 2003, and 2 in 2004. Juveniles were not included in every sampling.

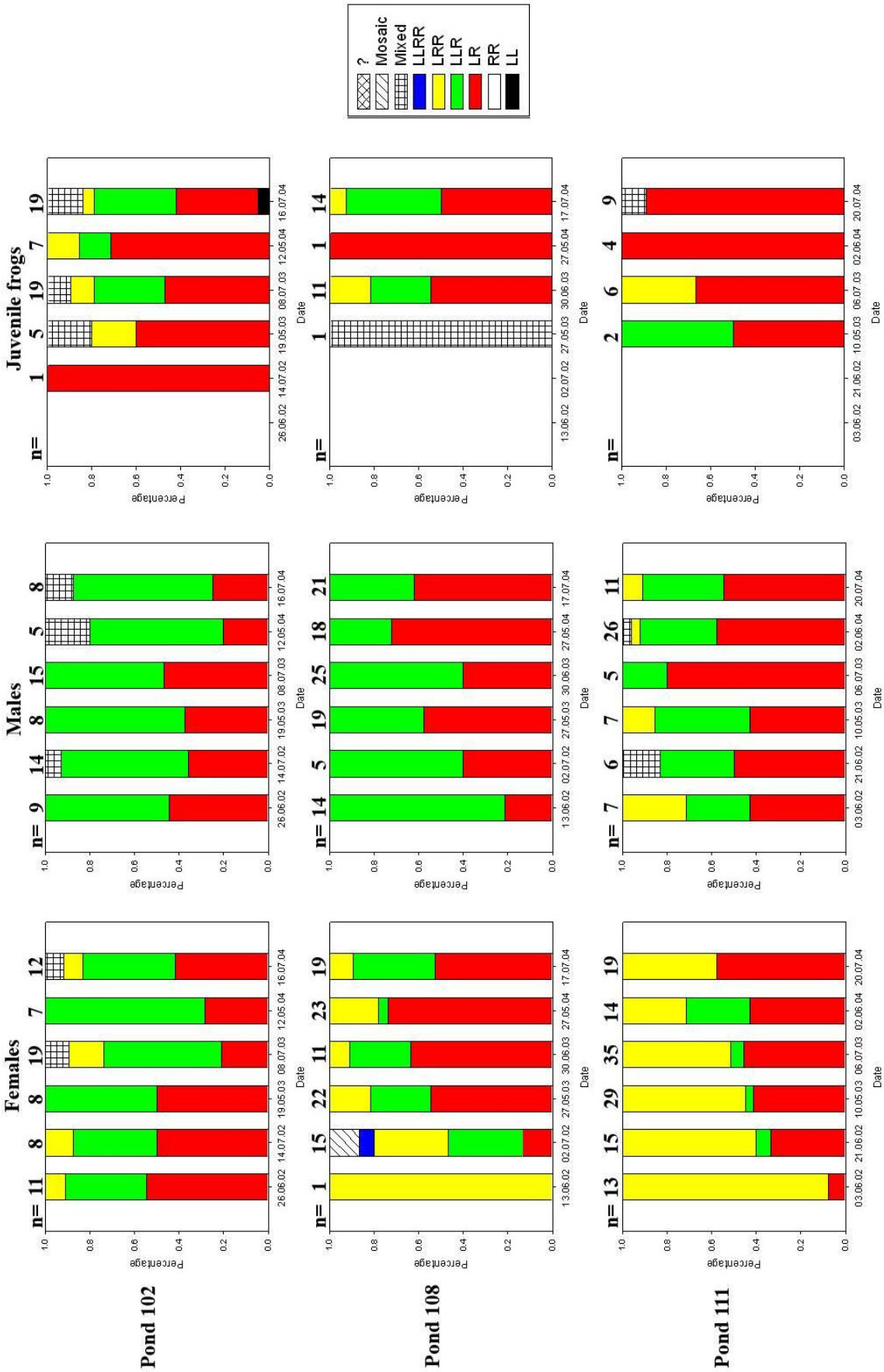


**Fig. 7:** Genotype composition of core ponds 102, 108 and 111, listed for females, males, and juveniles. Sampling occurred from 2002-2004 twice a year. Juveniles were not included in every sampling.

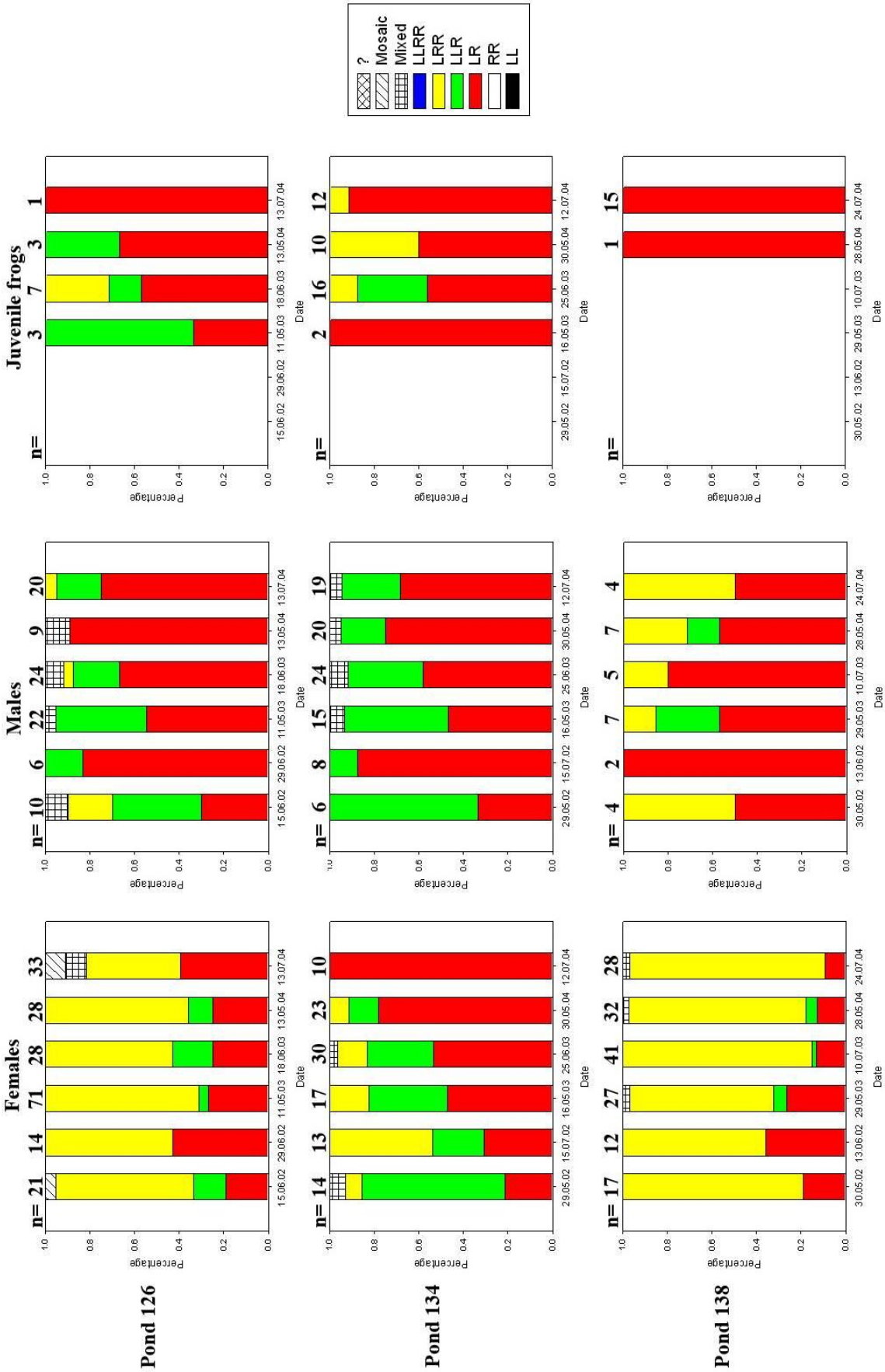


**Fig. 8:** Genotype composition of core ponds 126, 134 and 138, listed for females, males, and juveniles. Sampling occurred from 2002-2004 twice a year. Juveniles were not included in every sampling.

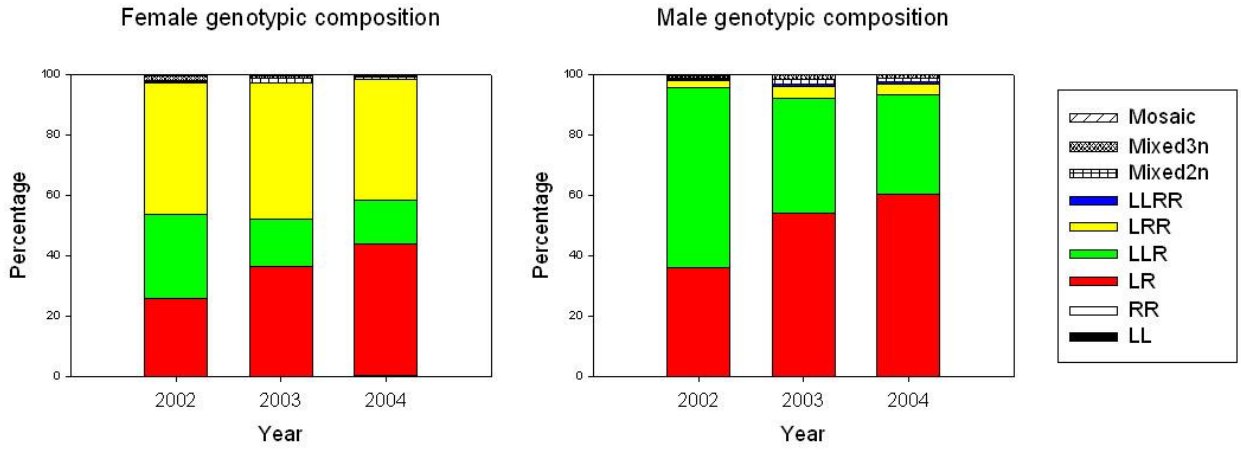


**Fig. 9:** Relative amounts of the different genotypes in females (left) and males (right), shown for 2002, 2003 and 2004 (pooled over all ponds). Whereas the amount of LRR animals in Skåne remains stable, LR animals gain in relative frequency at the cost of LLR animals in both sexes.

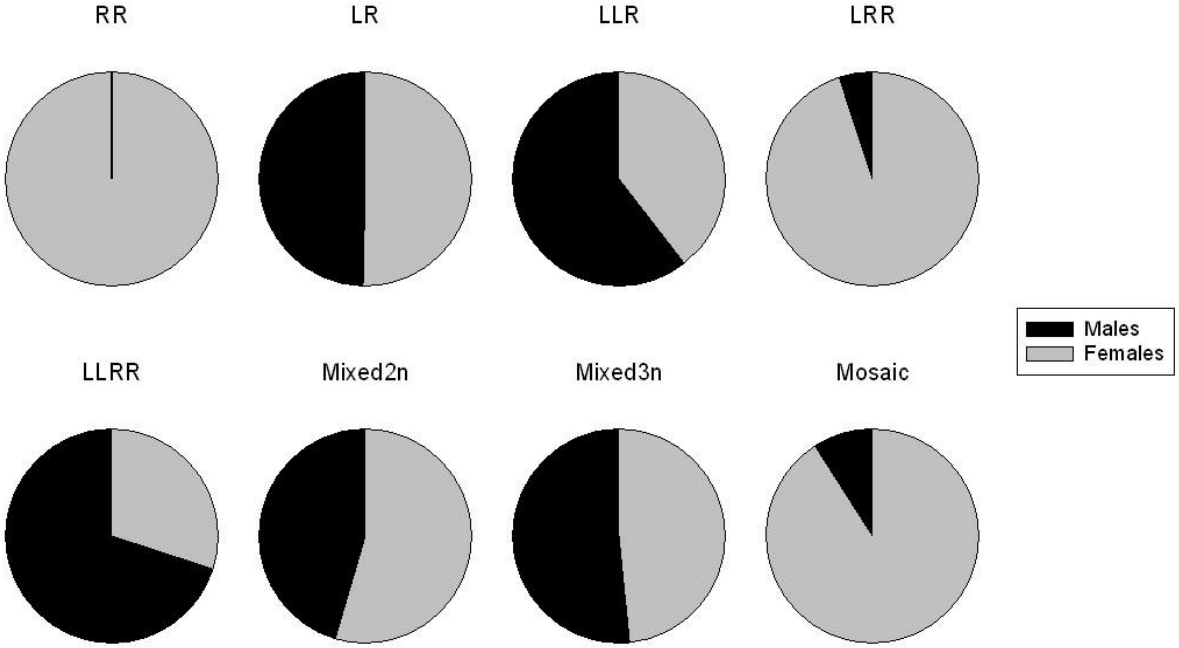


**Fig. 10:** Sex frequencies in different genotypes (pooled over all adult individuals, 2002-2004). Whereas LRR, RR and mosaic animals are almost exclusively female (grey), males (black) are dominant in LLRR and, to a lesser extent, also in LLR.

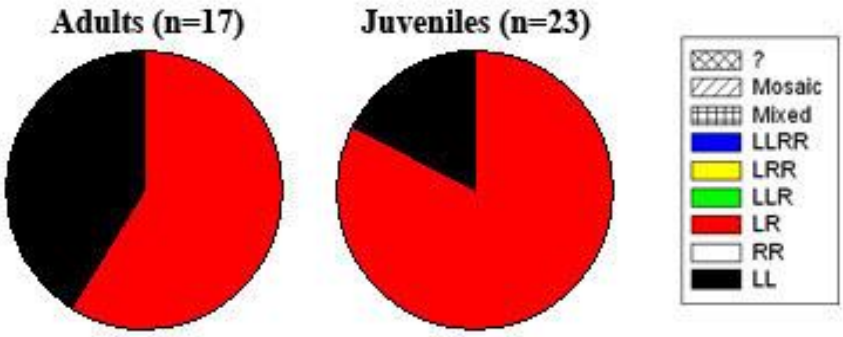


**Fig. 11:** Genotype composition of the Östergötland population. It clearly depicts an LE-population type, consisting only of LL and LR animals.

# CHAPTER 3

## The influence of ecological factors on the genotypic composition of pure *Rana esculenta* populations in Sweden

CHRISTIAN JAKOB & MARTINA ARIOLI

## Abstract

*Rana esculenta* (genotype LR) is a hybrid of the water frog species *R. lessonae* (LL) and *R. ridibunda* (RR). Reproducing hybridogenetically, it occurs in sympatry with one of its parental species throughout most parts of Europe, coexisting in the same habitat. It has been shown that *R. esculenta* differs in its ecological needs compared to its parental species, as well as *R. ridibunda* is differing in its requirements from *R. lessonae*. *R. esculenta* may form also pure hybrid populations, mainly along its northern distribution range. Triploid forms occurring in these pure hybrid populations are thought to take over the role of the parental species for reproduction. Previous investigations on pure hybrid populations in Southern Sweden have shown that genotype composition varies between ponds, but also changes between the years.

In this study, we have investigated one possible reason for the spatial and temporal structure of pure hybrid populations in Southern Sweden. The relative amount of diploid (LR) and triploid (LLR / LRR) animals in each population may be driven by ecological factors, with triploid animals exhibiting ecological needs similar to the parental species because one genome is present in double copy number.

In our study, *R. esculenta* proved tolerant for a wide range of physicochemical conditions. Relative genotype abundance was dependent on variables describing pond habitus. Diploid *R. esculenta* preferred larger ponds with more trees (less grassland, respectively). The smaller the pond and the higher the amount of roads, buildings and

other constructions near the pond, the higher the relative abundance of triploid LLR hybrids was. Changes in genotype compositions over the years did not correlate with changes in physicochemical conditions, although an influence of dissolved oxygen and temperature was noticeable.

*Keywords: Rana esculenta*, pure hybrid populations, hybridogenesis, ecology, genotype composition, triploid, water chemistry, pH, temperature, dissolved oxygen, Sweden

## Introduction

In zoology, hybrid animals were long considered "evolutionary dead-ends" (see review in Seehausen 2004). Hybrids tend to be less fit in parental habitats than their parental genotypes, although some hybrid genotypes may even be fitter under certain circumstances (Barton 2001). Additionally, F1-generation hybrids may exhibit heterosis (increasing their fitness above the parental level), but these effects normally get lost in subsequent generations (reviewed in Kearney 2005). Most F1 hybrids tend to be intermediate in character to the parental species (Grant and Grant 1992, Vences et al. 2003, Mavárez et al. 2006). The intermediate niche hypothesis (INH, in Moore 1977) postulates that these hybrids use the intermediate niches occurring in the area and can thus persist along with the parental species.

Once formed, many hybrids no longer reproduce sexually due to difficulties during gametogenesis (Bullini 1994, Dowling and Secor 1997). Examples are vertebrate parthenogens, gynogens, and hybridogens of hybrid origin that pass on their genome clonally.

In the European water frog system, the lake frog *Rana ridibunda* (genome composition RR) and the pool frog *Rana lessonae* (LL) can hybridize and produce the edible frog *Rana esculenta* (LR). *R. esculenta* is a bisexual taxon which is reproducing hybridogenetically, meaning that it discards one part of its genome (L or R) prior to meiosis from its germ line and transmits the other part clonally to the offspring. In wide parts of Europe, *R. esculenta* is therefore forced into sympatry with one of its parental species to regain the lost genome. Due to accumulation of deleterious mutations on the clonally transmitted genome, homotypic matings between *R. esculenta* result in inviable offspring, preventing backcrosses to the other parental genotype (Graf and Polls Pelaz 1989, Vorburger 2001a, b). In contrast to the above-mentioned sympatry, *R. esculenta* forms populations devoid of any of the parental species along the northern range of its distribution. These pure hybrid populations consist of diploid and polyploid (mostly triploid) animals. In previous investigations of pure hybrid populations in Southern

Sweden, animals of LR, LLR, LRR and LLRR genotype were found among the adults (Jakob et al., chapter 2 in this publication).

The ecological needs of the parental species *R. lessonae* and *R. ridibunda* differ mainly in habitat size and habitat structuring for the adults and in oxygen demands for larvae (summarized in Plötner 2005): *R. ridibunda* prefers larger water bodies such as oxbows, still river segments, channels, and eutrophic, shallow lakes which feature rich aquatic vegetation and are at least partially exposed to direct sunlight. *R. ridibunda* is susceptible to hypoxic conditions, and seems to be restricted to water bodies with pH > 6.5. *R. lessonae*, on the other hand, prefers vegetation-rich moor ponds, smaller ponds in forests or in meadows, and ditches. It occurs in water bodies with pH < 5.5, but also in slightly alkaline waters as long as there is a shallow water zone with abundant vegetation. *R. lessonae* larvae are also much more tolerant to hypoxic conditions and thrive better under warm temperatures (Negovetic et al. 2001).

In comparison to its parental species, the hybrid *R. esculenta* supported the intermediate niche hypothesis when it came to breeding habitat partitioning in a French river floodplain (Pagano et al. 2001), relative frequencies in relation to ecological conditions in Swiss ponds (Holenweg Peter et al. 2002), growth efficiency (Rist et al. 1997) and freezing tolerance (Voituron et al. 2005). However, when comparing predator tolerance (Anholt et al. 2005), feeding efficiency and growth rates (Rist et al. 1997), *R. esculenta* outperformed the other species. In an experiment done by Hotz et al. (1999), larval life-history traits (such as growth, time to and weight at metamorphosis) of F1-generation hybrids exhibited heterosis.

In pure hybrid populations, triploid animals of the LLR and LRR genotype are thought to adopt the role as sexual hosts which in mixed populations are taken on by the parental genotypes LL and RR, respectively. As investigations of Danish (Christiansen et al. 2005) and Swedish pure hybrid populations (Jakob et al., chapter 2 in this publication) have shown, the majority of such populations consist of varying amounts of diploid *R. esculenta* (LR), together with both LLR and LRR individuals. In the present study, we investigate in the area of Southern Sweden whether relative frequencies of the three

genotypes, and hence the genotypic composition of pure hybrid populations, depend on ecological factors as compositions of mixed *R. lessonae* / *R. esculenta* populations do (see Holenweg Peter et al. 2002). We have investigated both features potentially influencing the aquatic life stages of these frogs (water chemistry, physical parameters) and parameters that are likely to affect the adult, amphibian life stages (pond morphology, land use, climate). Genetic investigations of eggs and larvae have indicated that mating is happening randomly and all genotypes occur among tadpoles from all ponds (Arioli 2007). Differing population structures between ponds among adults may therefore exist due to differential survival of the genotypes caused by different ecological factors.

## Methods

Sampling was conducted in the years 2002-2004, during the months of May - August, in 12 "core" ponds located in South-Western Skåne (Scania), Southern Sweden. These ponds were sampled twice in 2002, and at least 2 times each in 2003 and in 2004. Additionally, 11 more ponds were only measured twice in 2002 (Fig. 1). Temperature and dissolved $O_2$ (DO) were measured with an Orion DO-meter model 820 (Thermo Electron Corp., Waltham, MA, USA) in 2002/2003, and an HQ20 LDO sensor (Hach-Lange GmbH, Hegnau, Switzerland) in 2004. An Orion pH-meter model 230A (Thermo Electron Corp., Waltham, MA, USA) was used in 2002 and 2003 to measure pH, in 2004 it was a Hach sensIon pH electrode on an HQ 20 (Hach-Lange GmbH, Hegnau, Switzerland). Water samples (500ml) were taken 1-4 times per season and stored at 4°C until analysis within 24h. Samples were filtered through LS 14 filter paper (Schleicher & Schuell GmbH, Dassel, Germany) with a waterjet pump, then processed with Dr. Lange LCK cuvette tests, and analyzed with a LASA 100 laboratory photometer (Hach-Lange GmbH, Hegnau, Switzerland). The following parameters were analyzed: water hardness including amounts of $Ca^{2+}$ and $Mg^{2+}$ (°d; LCK 327), total nitrogen ($TN_b$; LCK 138), total carbon, total organic carbon, total inorganic carbon (TC, TOC, TIC; LCK 380 & 381), and total phosphorous ($PO_4$-P; LCK 349).

In 2003 and 2004, automatic HOBO H8 temperature loggers (Onset Computer Corp., Cape Cod, MA, USA) were installed in all core ponds, measuring temperature hourly, 20 cm above and below the water line. Logger positions had to be adjusted several times during the season 2003, due to sinking water levels because of a dry spell. Due to vandalism, one logger had to be replaced in 2003 (pond 032), causing an interrupt in data collection; another logger was stolen in 2004 (pond 138), resulting in data loss.

In addition to the measurements of physical and chemical parameters, the ponds were also measured in terms of pond area and circumference, using GIS data and aerial photos in ArcView GIS 3.3 for Windows (© 1992-2002 ESRI Inc., Redlands, CA, USA). Land use in an area 5m, 20m and 100m around the ponds was estimated and assigned to 5 categories, named "wooded" (including forests, trees), "grassland" (meadows, pastures, lawns), "agriculture" (fallows, fields), "wetland" (water bodies, swamps) and "infrastructure" (roads, buildings). Also, the relative amount of pond coverage by submerged vegetation, floating vegetation (including reed) and canopy cover was estimated. Pond depths were estimated and ponds arbitrary classified as deep (>3m), medium (1.5 - 3m), or shallow (<1.5m).

Temperature (daily minimum, maximum and mean) and daily precipitation data for 2000-2004 in the sample region (observation station Malmö A) and reference normal data for 1961-1990 were licensed from the Swedish Meteorological and Hydrological Institute (SMHI, Norrköping, Sweden).

Frog sampling (conducted twice a year at each sampled pond) and genotyping based on microsatellite loci dosage effects and flow cytometry were performed as described by Jakob and Arioli (chapter 1 in this publication). For this study, only adult frogs of the three most frequent genotypes (LR, LLR and LRR) were used. The relative genotype frequencies for each pond are shown in Table 1.

### *Data preparation and statistical analysis*

Percentage data was arcsine square-root transformed before statistical analysis ($x' = \arcsin\sqrt{x}$) to assure a nearly-normal distribution. Relative DO data was an

exception because these data were ranging above 100% and showing a normal distribution. Non-normally distributed pond variables were log-transformed ($x' = \log(x+1)$) to improve normality of the data set (applied to water hardness including amounts of $Ca^{2+}$ and $Mg^{2+}$, TC, TIC, TOC, $TN_b$, $PO_4$-P, area and circumference) and area and circumference were standardized (mean=0, std dev=1) sensu Quinn and Keough (2004).

Data describing pond habitus (area, circumference, amount of submerged and floating vegetation, tree cover) and land use (estimated 5, 20 and 100m around the pond) were then submitted to a principal component analysis (PCA, PROC FACTOR in SAS) with an orthogonal varimax rotation to reduce the number of variables. Factors with an eigenvalue ≥ 0.97 were retained. Two further PCAs were performed, one on the pooled data from 2002-2004 for 12 parameters of water chemistry and physics (relative and absolute DO, pH, temperature, $TN_b$, $PO_4$-P, total water hardness including $Ca^{2+}$ and $Mg^{2+}$, TC, TIC, TOC) and the other for 8 logged temperature parameters, 4 each for water and air from 2003 and 2004 (daily mean, minimum, maximum and span). For each of the resulting factors, variables with factor scores >0.71 were considered as main describing variables following Comrey and Lee (1992) who rate scores >0.71 as "excellent". Finally, one representative value was selected for each factor out of the main describing variables, based on high factor scores (>0.84) and biological significance. These representatives allow an easier understanding of the results than factor scores and yielded in subsequent statistical analyses qualitatively the same results as analyses based on scores.

To investigate relationships between ecological variables and genotype composition, stepwise linear regressions were performed (PROC REG in SAS) for ponds sampled in July 2002 (n=22, omitting 032A from the sample), using the (transformed) relative amounts of LR and LLR genotypes as dependent variables and the representatives of PCA factors, pond coordinates and classifications of pond depth as explanatory variables. Starting from a full model, backward elimination was performed with a significance value of <0.1 for retaining variables.

To test for a correlation of yearly changes of relative genotype abundances and physicochemical variables, linear regressions (PROC REG in SAS) were performed with data from 11 core ponds (omitting 032A from the sample). The changes in LR and LLR abundance from 2002 to 2004 were tested against the changes in the above mentioned representative water parameters over the same period. Differences were standardized (mean=0, std dev=1) for all variables before analysis.

Statistical analyses were carried out with SAS 9.1.3 SP3 for Windows (© 2002-2003 SAS Institute Inc., Cary, NC, USA.). Graphs were produced using SigmaPlot 2002 v8.02 for Windows (© 1986-2001 SPSS Inc., Chicago, IL, USA).

## Results

### *Principal component analyses*

PCA of 20 descriptive pond variables as described in the material and methods section resulted in 6 factors accounting for 89% of the total variation. They can roughly be described as (1) forested area (which correlates negatively with grassland area), (2) pond size, (3) human impact, (4) distant wetland, (5) floating vegetation and (6) nearby wetland (see Table 2). For subsequent analyses, the following variables were chosen to represent these factors: (1) relative amount of forest area 20m around the pond, (2) log pond circumference, (3) relative amount of infrastructure area 20m around the pond, (4) relative amount of wetland area 100m around the pond, (5) floating vegetation and reed coverage, and (6) relative amount of wetland area 5m around the pond. After excluding two parameters because of excess of zero values (relative amounts of agriculture and of wetland 5m around the pond, respectively), the PCA resulted in the same order and qualitative weighing of the first 5 factors.

PCA of 12 physicochemical pond variables as described in the materials and methods section delivered 4 factors, explaining 87% of the variation in the dataset. They can be described as (1) inorganic contents, (2) nutrients, (3) DO and pH, and (4) temperature (see Table 3). The selected representatives for these factors were (1) log water hardness,

(2) log TOC, (3) absolute DO, and (4) temperature. When testing these representatives for differences between ponds, years and months (MANOVA, PROC GLM in SAS), they all differed among ponds (Fig. 2 and Table 4). Temperature also differed significantly between month and years (Table 4), which is illustrated in Fig. 3 with mean logged temperature data of June and July 2003 and 2004.

PCA of the 8 temperature variables derived from logged data produced 2 factors, accounting for 89% of variation in the data. These factors described (1) temperature values and (2) temperature fluctuation (see Table 5). As representative variables, daily mean water temperature and daily water temperature span were selected. When tested for correlation with the single temperature measurements taken during physicochemical water sampling (PROC CORR in SAS), both representatives correlated highly with these in both years (2003: N=33, $p(T_{mean}) < 0.0001$, $p(T_{span}) < 0.05$; 2004: N=22, $p(T_{mean}) < 0.05$, $p(T_{span}) < 0.0001$. Therefore, we used the single measurements rather than the logged temperature data in MANOVAs which included physicochemical water data.

*Regressions*

Because $O_2$ levels in pond 032A dropped below 5% and neither egg clutches, nor tadpoles, nor newly metamorphosed frogs were encountered during our investigations (Arioli and Jakob, chapter 6 in this publication), the pond was excluded from further analyses. A stepwise linear regression model related the relative numbers of LR genotypes (and, hence, the proportions of diploid and triploid animals) in the remaining 22 ponds to pond depth, pond area, the 5 main representative pond habitus variables and the 4 representative physicochemical variables in July 2002 (when data were available for all ponds). This stepwise linear regression resulted in a highly significant model (df=3, F=5.22, p<0.01). The proportion of diploids increased significantly with pond size (F=7.01, p=0.02) and the amount of forested area (F=10.15, p<0.01) (Fig. 4), and tended to increase with the amount of wetland nearby, i.e., within 5m (F=3.16, p=0.09). A second stepwise linear regression model, relating the proportion of LLR genotypes in 2002 to the same variables also resulted in a highly significant model

(df=3, F=5.93, p<0.01). LLR proportions decreased significantly from east to west (longitudinal coordinate, F=6.88, p=0.02). Relative LLR amounts tended to decrease with pond size (F=4.35, p=0.052) and to increase with human infrastructure within 20m (F=3.05, p<0.1). The results were largely influenced by pond 138, an outlier with respect to its geographical position far west outside of our principal sampling area (Fig. 1) and its population composition (no LLR frogs were caught in 2002). When pond 138 is excluded from the analysis, the resulting model (df=2, F=5.43, p=0.01) contained only pond size (F=5.68; p=0.03) and human influence (F=6.66, p<0.02) as significant effects on LLR proportions, as shown in Fig. 5.

When analyzing correlations of yearly changes in genotype abundances and in physicochemical water parameters of 11 core ponds between 2002 and 2004 by performing linear regressions, no significant results were found. However, some trends were apparent. An increase in LR genotypes was associated with an increase in DO (t=1.44, p=0.16), whereas LLR changes tended to decrease with increasing DO differences (t=-1.87, p=0.08) and to increase with temperature changes (t=1.90, p=0.08).

## Discussion

Pond 032A was excluded from analysis because of its low oxygen levels in the course of the breeding season and the apparent lack of reproduction in this pond (no eggs, tadpoles, or metamorphs encountered) (Arioli 2007). This pond can either be seen as a sink population if breeding is attempted at the site, or as a “stepping stone” for animals migrating between the forest and nearby breeding ponds located at a 100m distance in a meadow.

When explaining the proportion of LR and LLR genotypes in 22 ponds sampled in July 2002 with habitus, physicochemical variables and geographical location by stepwise regression, the resulting models were highly significant. The remaining variables in the model were only habitus variables, all physicochemical variables were eliminated from the final model. The amount of LR genotypes in these ponds was significantly positively

influenced by pond circumference and amount of forested area (Fig. 4). A slight positive influence by directly adjacent water bodies could be determined, but as this category was >0 only for one pond, it should be put aside for final conclusions. In short, diploid *R. esculenta* preferred larger ponds with more trees (less grassland, respectively). For triploid *R. esculenta*, pond 138 was excluded from the analysis because of its outlier status. The amount of LLR correlated negatively with pond size and positively with the amount of human impact (Fig. 5). In short: the smaller the pond and the higher the amount of roads, buildings and other constructions near the pond, the higher the relative abundance of triploid LLR hybrids. In their preference for smaller water bodies, LLR is similar to LL. There was no preference for LLR towards forest ponds, but the other triploid genotype LRR showed, comparable to RR, a preference for open ponds exposed directly to the sun. We could not show any significant influence of physicochemical parameters on genotype abundance. Neither pH, nor $O_2$, which for LL and RR genotypes seem to be of high importance, especially during larval stages (Plénet et al. 2000, Holenweg Peter et al. 2002, Plötner 2005), were affecting hybrid genotype frequencies.

In general, pure *R. esculenta* populations showed tolerance for a wide range of ecological conditions. For example, pH ranged from mildly acid (pH 5.35 in pond 102) to highly alkaline (pH 12.2 in pond 111). Distributions of the 4 representative variables describing physicochemical pond properties are shown in Fig. 6. Also in previous investigations, a high tolerance of *R. esculenta* towards environmental disturbance and towards pollution was shown, compared to its parental species *R. lessonae* (Fioramonti et al. 1997, Bucci et al. 2000) which was attributed to the broader adaptation of the hybrid. Even the possible use of the relative abundance of *R. esculenta* in LE-systems as bioindicator for polluted waters was suggested in an Italian publication (Andreani et al. 2003), as *R. esculenta* are still present under conditions where their parental species *R. lessonae* is absent. We observed also a high tolerance for predator presence, as virtually every pond in our sample except 032A was stocked with fish; also, crayfish were common (C. Jakob and M. Arioli, pers. obs.). The ability of hybrid tadpoles to bear high fish predator tolerance was also reported previously by Anholt et al. (2005).

Given this broad tolerance, it may be that the span of physicochemical conditions in our ponds was too narrow and that only ponds with more extreme conditions would differ clearly in their LR, LLR and LRR proportions. On the other hand, a potential heterosis effect in hybrids may mitigate any L- and R-specific susceptibility to physicochemical parameters that may exist in the parental species. Moreover, the ecological conditions may not influence triploid animals in the same way as parental species. As it was shown for example in cotton plants, polyploidy does not always lead to enhanced transcription of duplicated genes (Adams et al. 2003). To put it in a more concise way: One and one does not always equal two (Otto 2003), so that 2 copies of one genome don't necessarily shift the ecological needs of the hybrid in the direction of the respective parental species. Consequently, there were no significant changes in physiochemical water parameters that could explain the changes in relative genotypic abundance between the years 2002 to 2004, although temperature and DO seem to have at least a partial influence. This result has to be treated with caution, however. First, such a short time span does not allow distinguishing between stochastic fluctuations and a direct causal influence of environmental variables. Second, effects on for instance larval survival will show on a population level only some years after the recording of the variables in question. On the other hand, climate during overwintering may have a direct influence on adult survival and affect genotypes differently. However, it is not known to date if LLR and LRR differ in their overwintering behaviour similar to their parental species *R. lessonae* and *R. ridibunda* (Berger 1982, Holenweg and Reyer 2000, Voituron et al. 2005). As SMHI climate data for southern Sweden (Malmö A) show, there was a tendency for higher temperatures between 2000 and 2004, especially for warmer winters compared to the 30-year normal data (Fig. 7). For precipitation, there were no apparent trends as there were alternating wet and dry years compared to the longtime mean. If any, springs were tending to be dryer than on average (Fig. 8). The effects of such climatic conditions on *R. esculenta* genotypes have to be studied in greater detail to relate them also to the recent range expansion of *R. esculenta* in Skåne (J. Pröjts, pers. comm.; J. Loman, pers. comm.). As a last consideration, offspring genotype and ploidy are dependent directly on the genotypic combination of their parents (see Table

6). There are strong indications for indiscriminate mating (Günther and Plötner 1990, Schmeller et al. 2005, Arioli 2007). Therefore, changes in genotypic frequencies may be dependent directly on previous population composition and may be driven by stochastic events (see also the model by Som and Reyer 2006).

## Acknowledgements

We would like to thank Sandra Röthlisberger for her help with lab work. The group of Rolf Zinkernagel at Zürich University Hospital provided us access to their FACSCalibur. Bo Baldetorp of Lund University Hospital and Eva Niederer of the Institute for Biomedical Engineering at the ETH Zürich provided practical and technical advices for flow cytometric analysis. Jon Loman of Lund University took care of all our Swedish administrative matters. Our supervisor H.-U. Reyer made the project possible and provided valuable input for statistical problem-solving. He also commented on the manuscript.

The work was funded by the Swiss National Science Foundation, grant No. 3100-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-18591-02, 522-9286-03, 522-6571-04) and by Länsstyrelsen Östergötland (522-1923-04).

## Author contributions

C.J. and M.A. contributed equally to this work. Both authors carried out all field- and lab work together. CJ performed statistical analyses and wrote the paper. Both authors discussed the results and MA commented on the manuscript.

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## Tables

**Table 1:** Proportions of genotypes in 23 sampled ponds. Only adult frogs of main genotypes (LR, LLR and LRR) are listed.

| | 2002 | | | 2003 | | | 2004 | | |
|---|---|---|---|---|---|---|---|---|---|
| Pond | LR | LLR | LRR | LR | LLR | LRR | LR | LLR | LRR |
| 001 | 0.0769 | 0.8974 | 0.0256 | 0.3023 | 0.6977 | 0.0000 | 0.2703 | 0.6486 | 0.0676 |
| 010 | 0.3256 | 0.3488 | 0.2791 | | | | | | |
| 011 | 0.2128 | 0.4255 | 0.3404 | 0.6552 | 0.1609 | 0.1264 | 0.6585 | 0.2520 | 0.0894 |
| 012 | 0.2432 | 0.2973 | 0.4595 | | | | | | |
| 014 | 0.2093 | 0.5581 | 0.2093 | 0.5875 | 0.2375 | 0.1500 | 0.6575 | 0.3014 | 0.0411 |
| 021 | 0.4286 | 0.4694 | 0.1020 | | | | | | |
| 023 | 0.3023 | 0.5814 | 0.1163 | | | | | | |
| 024 | 0.3438 | 0.6250 | 0.0313 | | | | | | |
| 032 | 0.5306 | 0.3673 | 0.1020 | 0.4545 | 0.4364 | 0.0909 | 0.5618 | 0.2472 | 0.1685 |
| 032A | 0.3333 | 0.2353 | 0.3922 | 0.3387 | 0.1290 | 0.5323 | 0.4912 | 0.3158 | 0.1404 |
| 089 | 0.2500 | 0.0119 | 0.7143 | 0.3933 | 0.1267 | 0.3933 | 0.4421 | 0.1053 | 0.4421 |
| 101 | 0.4884 | 0.4419 | 0.0233 | | | | | | |
| 102 | 0.4524 | 0.4762 | 0.0476 | 0.3600 | 0.5400 | 0.0600 | 0.3125 | 0.5625 | 0.0313 |
| 108 | 0.2000 | 0.5429 | 0.1714 | 0.5195 | 0.4156 | 0.0649 | 0.6543 | 0.2593 | 0.0864 |
| 108A | 0.2927 | 0.4634 | 0.1707 | | | | | | |
| 111 | 0.2927 | 0.1220 | 0.5610 | 0.4605 | 0.0921 | 0.4474 | 0.5429 | 0.2429 | 0.2000 |
| 112 | 0.2381 | 0.5714 | 0.1667 | | | | | | |
| 123 | 0.1429 | 0.8571 | 0.0000 | | | | | | |
| 126 | 0.3529 | 0.1569 | 0.4510 | 0.3724 | 0.1517 | 0.4552 | 0.4778 | 0.0778 | 0.3667 |
| 134 | 0.3902 | 0.4146 | 0.1707 | 0.5233 | 0.3488 | 0.0814 | 0.7778 | 0.1667 | 0.0278 |
| 135 | 0.1389 | 0.6389 | 0.1667 | | | | | | |
| 137 | 0.1579 | 0.8421 | 0.0000 | | | | | | |
| 138 | 0.2571 | 0.0000 | 0.7429 | 0.1875 | 0.0375 | 0.7625 | 0.1127 | 0.0282 | 0.8310 |

**Table 2:** Rotated factor pattern resulting from principal component analysis with orthogonal varimax rotation of 20 variables describing pond habitus. Main describing variables of each factor are shown on grey background. Chosen representative variables are printed in bold italics. Categories of land use: 1: wooded, 2: grassland, 3: agriculture, 4: wetland, 5: infrastructure; estimated 5m, 20m, and 100m around the pond.

| Variable | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Factor 5 | Factor 6 |
|---|---|---|---|---|---|---|
| ***Category 1 (20m)*** | ***0.95630*** | -0.07193 | -0.13011 | -0.07638 | 0.18639 | -0.00122 |
| Category 1 (5m) | 0.95191 | -0.11982 | -0.17951 | -0.04383 | 0.09955 | -0.07791 |
| Category 1 (100m) | 0.94905 | 0.06695 | 0.03747 | -0.21508 | -0.00346 | -0.02977 |
| Amount of canopy coverage | 0.51670 | -0.15811 | 0.31990 | -0.26100 | 0.49020 | -0.25058 |
| Category 2 (100m) | -0.77075 | -0.52018 | -0.01644 | -0.23759 | 0.06224 | 0.03584 |
| Category 2 (5m) | -0.91222 | -0.03930 | -0.21005 | 0.26047 | -0.05099 | -0.00683 |
| Category 2 (20m) | -0.96257 | -0.04392 | 0.05402 | -0.05947 | -0.19199 | -0.01119 |
| ***Log Pond circumference (m)*** | 0.12744 | ***0.84765*** | 0.30680 | -0.07132 | -0.20844 | -0.19542 |
| Log Pond area ($m^2$) | 0.10744 | 0.81258 | 0.24704 | 0.00352 | -0.28781 | -0.26194 |
| Amount of submerged vegetation | 0.19204 | 0.67037 | -0.06544 | 0.18204 | 0.38644 | 0.41240 |
| Category 3 (100m) | -0.38100 | 0.66932 | -0.28424 | 0.40717 | -0.01968 | 0.00534 |
| Category 3 (20m) | -0.40369 | 0.48919 | -0.30895 | 0.40596 | -0.04063 | -0.08025 |
| ***Category 5 (20m)*** | -0.00443 | 0.00787 | ***0.96631*** | 0.05505 | 0.06397 | -0.03316 |
| Category 5 (100m) | -0.03825 | 0.02301 | 0.96314 | 0.05279 | 0.00908 | -0.07616 |
| Category 5 (5m) | -0.10858 | 0.32251 | 0.74727 | -0.40951 | -0.09381 | 0.08964 |
| ***Category 4 (100m)*** | -0.06298 | 0.01046 | 0.18117 | ***0.93495*** | -0.11841 | -0.09801 |
| Category 4 (20m) | -0.17966 | 0.22366 | -0.23655 | 0.72137 | 0.06730 | 0.22084 |
| ***Amount of floating vegetation*** | 0.23547 | -0.18388 | -0.00609 | -0.03053 | ***0.91683*** | 0.01753 |
| ***Category 4 (5m)*** | -0.07029 | -0.20120 | -0.02326 | 0.01075 | -0.02838 | ***0.92404*** |
| Category 3 (5m) | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| Explained variance | 5.82373 | 3.06759 | 3.04506 | 2.18786 | 1.47886 | 1.28201 |
| Cumulative proportion | 0.3319 | 0.5191 | 0.6910 | 0.7726 | 0.8357 | 0.8887 |

**Table 3:** Rotated factor pattern resulting from principal component analysis with orthogonal varimax rotation of 12 physicochemical water variables. Main describing variables of each factor are shown on grey background. Chosen representative variables are printed in bold italics. Percentage values were arcsine square-root transformed prior to analysis.

| Variable | Factor 1 | Factor 2 | Factor 3 | Factor 4 |
|---|---|---|---|---|
| ***Log Water hardness (°d)*** | ***0.97259*** | -0.17946 | -0.00737 | -0.03162 |
| Log TIC (mg/l) | 0.94210 | -0.00178 | -0.13696 | -0.11828 |
| Log $Ca^{2+}$ (mg/l) | 0.93454 | -0.20163 | 0.02755 | -0.10230 |
| Log $Mg^{2+}$ (mg/l) | 0.89116 | -0.11647 | -0.05062 | 0.03867 |
| Log TC (mg/l) | 0.67782 | 0.66095 | -0.17555 | -0.03884 |
| ***Log TOC (mg/l)*** | 0.04156 | ***0.87090*** | -0.12785 | 0.01925 |
| Log total nitrogen ($TN_b$, mg/l) | -0.25819 | 0.83273 | 0.10898 | -0.15058 |
| Log total phosphate ($PO_4$-P, mg/l) | -0.25953 | 0.78271 | -0.04806 | 0.09708 |
| ***DO (mg/l)*** | -0.20851 | -0.06870 | ***0.93386*** | 0.01699 |
| DO (%) | -0.23632 | -0.07093 | 0.92404 | 0.12742 |
| Log pH | 0.27015 | 0.00079 | 0.80627 | 0.01753 |
| ***T (°C)*** | -0.10784 | -0.01774 | 0.11330 | ***0.98018*** |
| Explained variance | 4.28014 | 2.59788 | 2.47234 | 1.03850 |
| Cumulative proportion | 0.3748 | 0.6136 | 0.7848 | 0.8657 |

**Table 4:** Results of a MANOVA relating variables that represent the physicochemical factors from the PCA (Table 3) to pond, year and month. Significant p values <0.05 are displayed in bold letters. N=97.

| | Log water hardness | | | Log TOC | | | Relative DO | | | T | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source | df | F | p | df | F | p | df | F | p | df | F | p |
| Year | 2 | 0.43 | >0.1 | 2 | 2.35 | >0.1 | 2 | 0.15 | >0.1 | 2 | 3.35 | **<0.05** |
| Month | 3 | 1.82 | >0.1 | 3 | 1.38 | >0.1 | 3 | 0.91 | >0.1 | 3 | 7.42 | **<0.0001** |
| Pond | 11 | 240.29 | **<0.0001** | 11 | 8.47 | **<0.0001** | 11 | 4.58 | **<0.0001** | 11 | 5.25 | **<0.01** |

**Table 5:** Rotated factor pattern resulting from principal component analysis with orthogonal varimax rotation of 8 daily temperature variables derived from logger data. Main describing variables of each factor are shown on grey background. Chosen representative variables are printed in bold italics. Percentage values were arcsine square-root transformed prior to analysis.

| Variable | Factor 1 | Factor 2 |
|---|---|---|
| Minimum water | 0.92692 | 0.07438 |
| Mean air | 0.92078 | 0.24614 |
| Minimum air | 0.87455 | -0.37627 |
| ***Mean water*** | ***0.87259*** | 0.41454 |
| Maximum water | 0.72775 | 0.63344 |
| Maximum air | 0.67115 | 0.65268 |
| Span air | -0.04337 | 0.94332 |
| ***Span water*** | 0.15290 | ***0.87293*** |
| Explained variance | 4.23860 | 2.85862 |
| Cumulative proportion | 0.6297 | 0.8872 |

**Table 6:** Offspring genotypes, depending on parental genotypes. Genotypes and ploidy are prevalently changing between generations. Homogenic offspring (LL, RR) are not viable and are shown on grey background. Diploid females can produce haploid and diploid eggs.

| F \ M | LLR | LR | LRR |
|---|---|---|---|
| LLR | - (LL) | LR | LR |
| LR | LLR LR | - (RR) LRR | - (RR) LRR |
| LRR | LR | LR | - (RR) |

## Figures

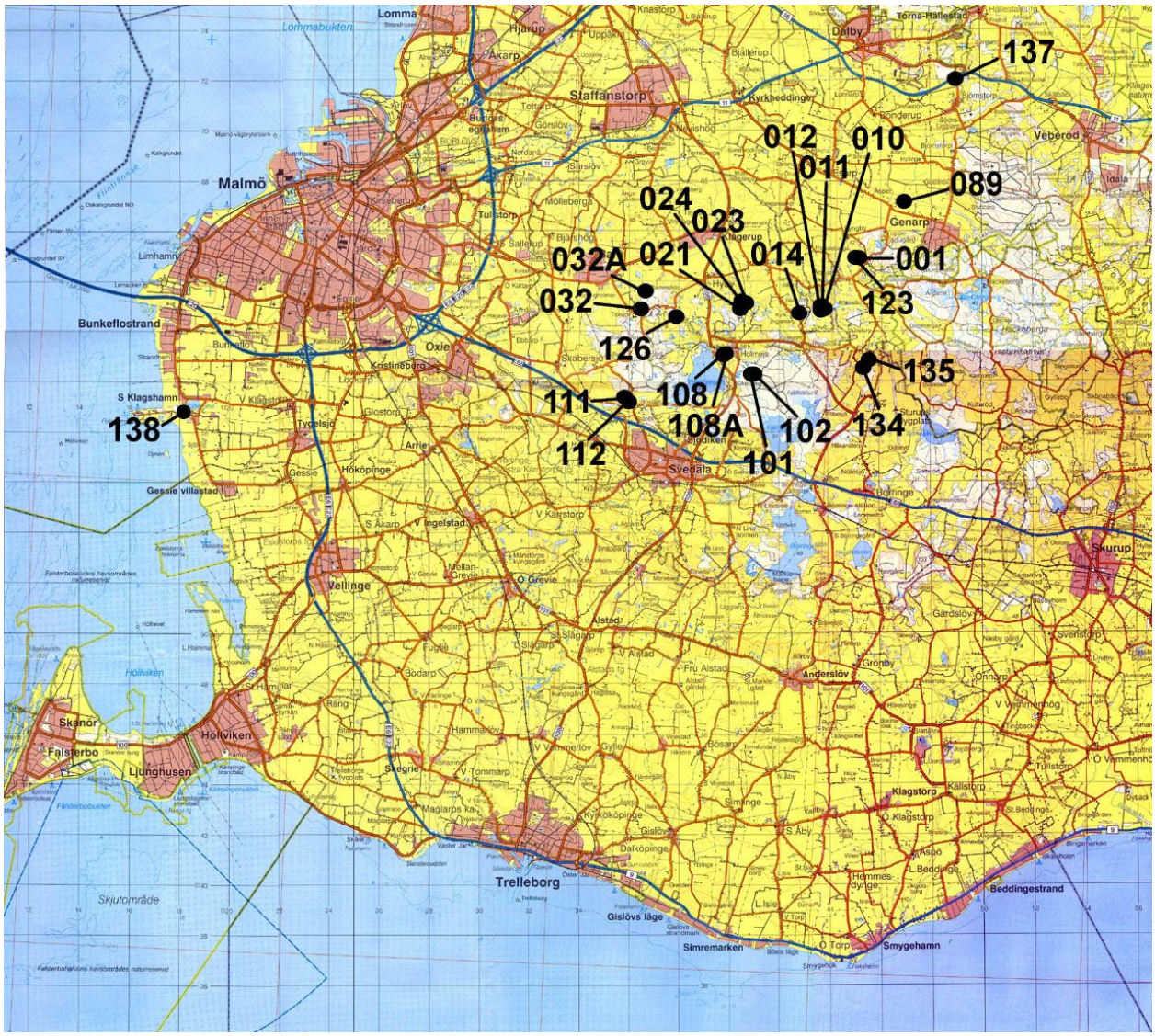


**Fig. 1:** Location of the 23 sampled ponds in southern Sweden. All ponds were sampled twice in 2002. Additionally, ponds 001, 011, 014, 032, 032A, 089, 102, 108, 111, 126, 134 and 138 ("core ponds") were sampled in 2003 and 2004 as well. Map: Adapted from Blå kartan, blad 31, edition 3 (Reproduction permission and © Lantmäteriverket Gävle 2006. Grant I 2006/1863).

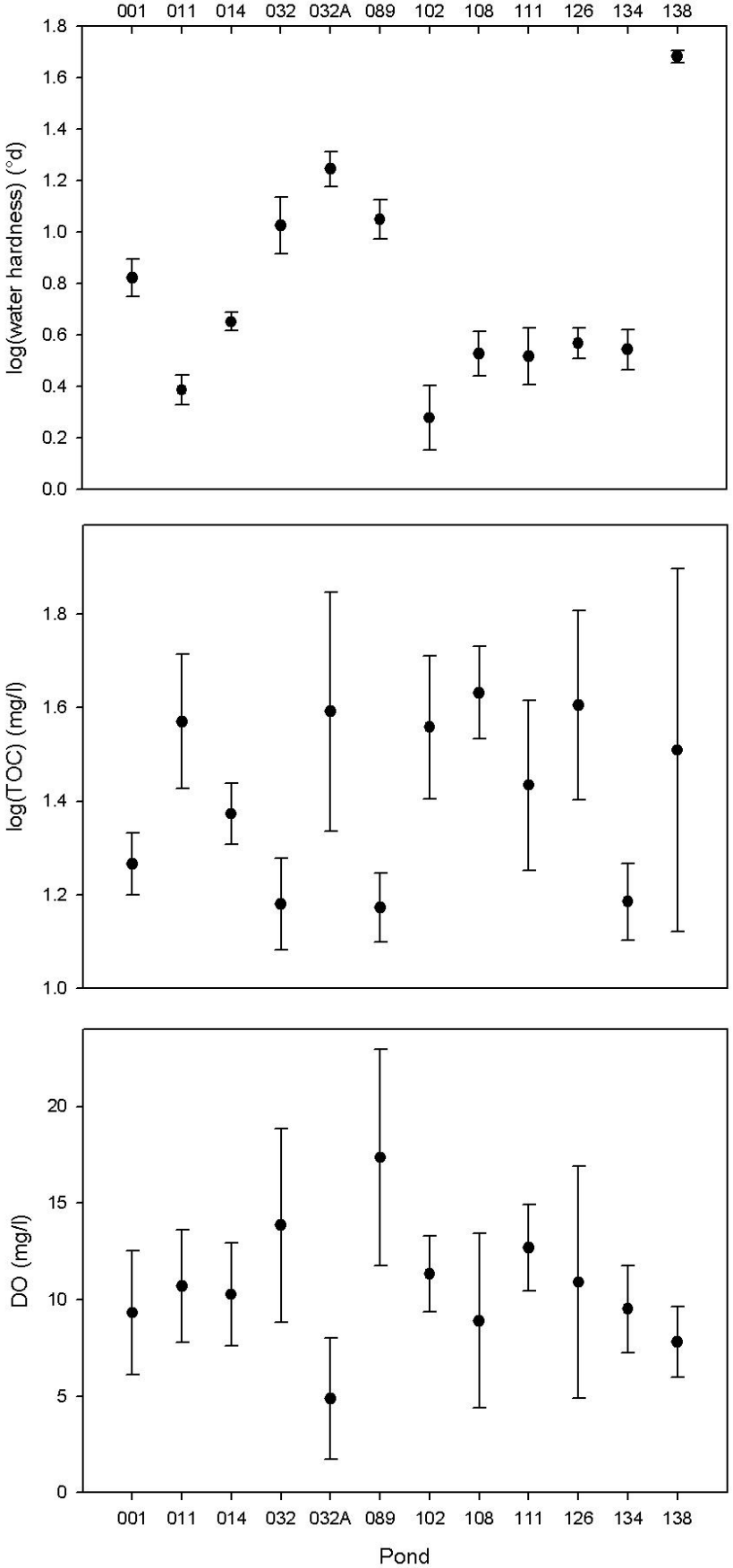


**Fig. 2:** Differences between the 3 representative water chemistry variables for the 12 core ponds. Top: log water hardness; Middle: log TOC; Bottom: absolute DO. Mean values for 2002-2004, error bars represent standard deviation.

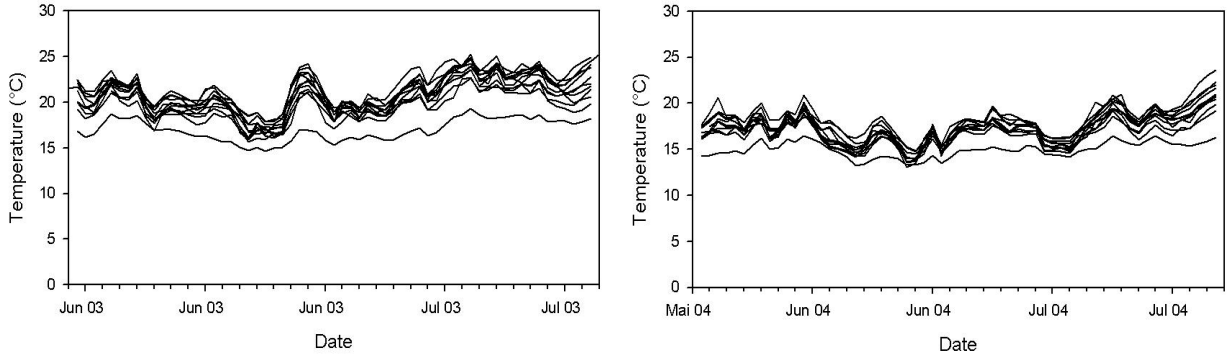


**Fig. 3:** Mean daily logged water temperature at 20cm below the surface for 12 core ponds in June and July 2003 (left) and for 11 core ponds (without pond 138) in May, June and July 2004 (right). Note the similar course of temperature for all ponds, except for pond 032A which was markedly colder in both years (lowest line in both graphs).

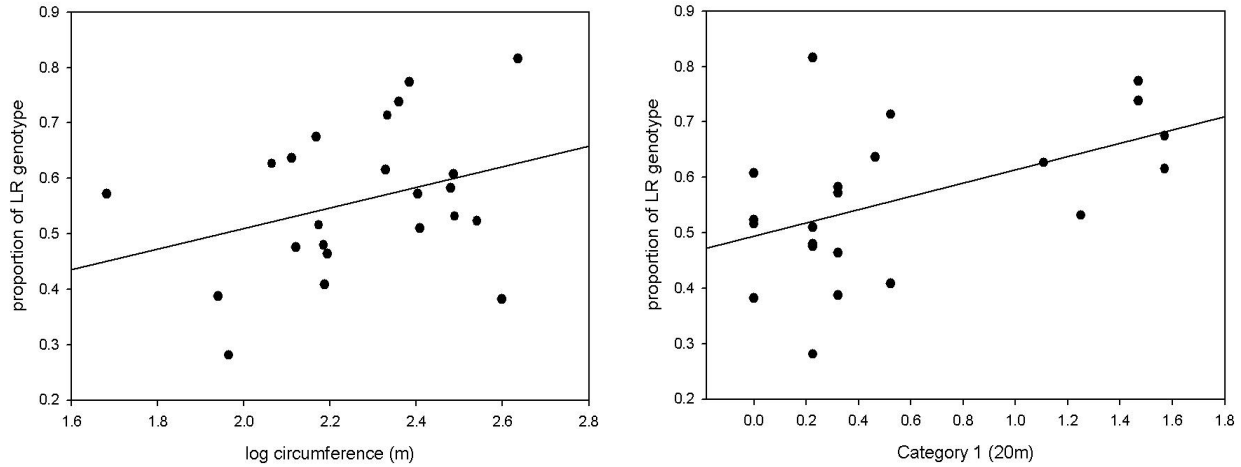


**Fig. 4:** Linear regressions for the proportions of LR genotypes in relation to pond size (left) and amount of forested area within 20m around the pond (right).

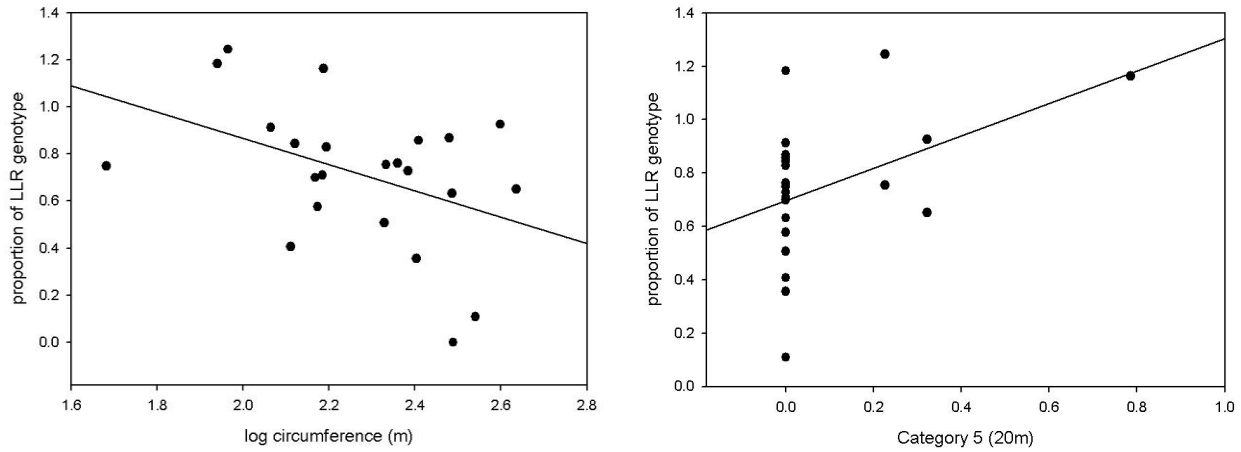


**Fig. 5:** Linear regressions for the proportions of LLR genotypes in relation to pond size (left) and amount of human influence within 20m around the pond (right).

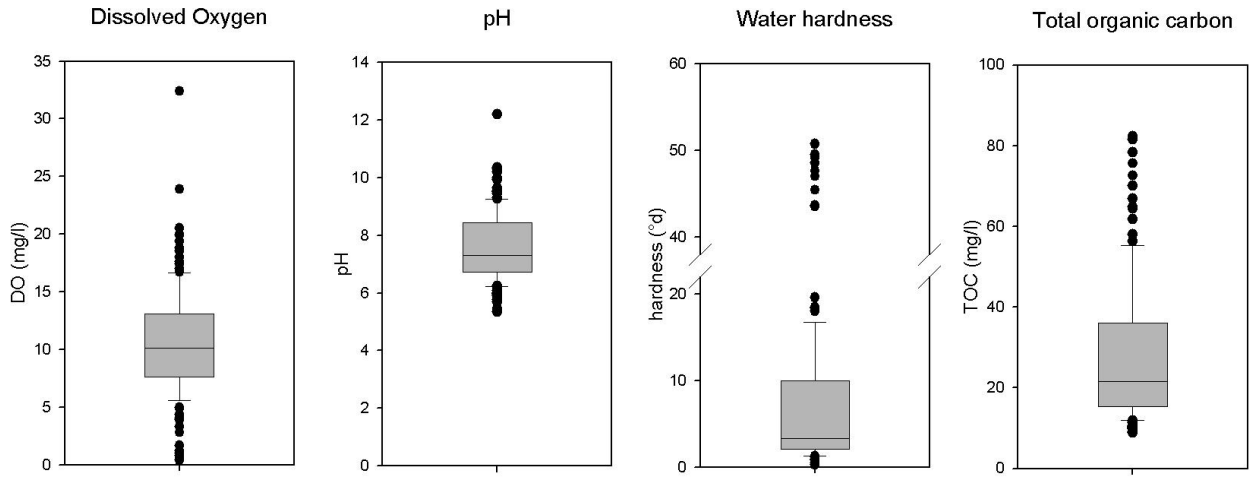


**Fig. 6:** Range of 4 representative variables for PCA factors defining physicochemical pond properties for 23 ponds.

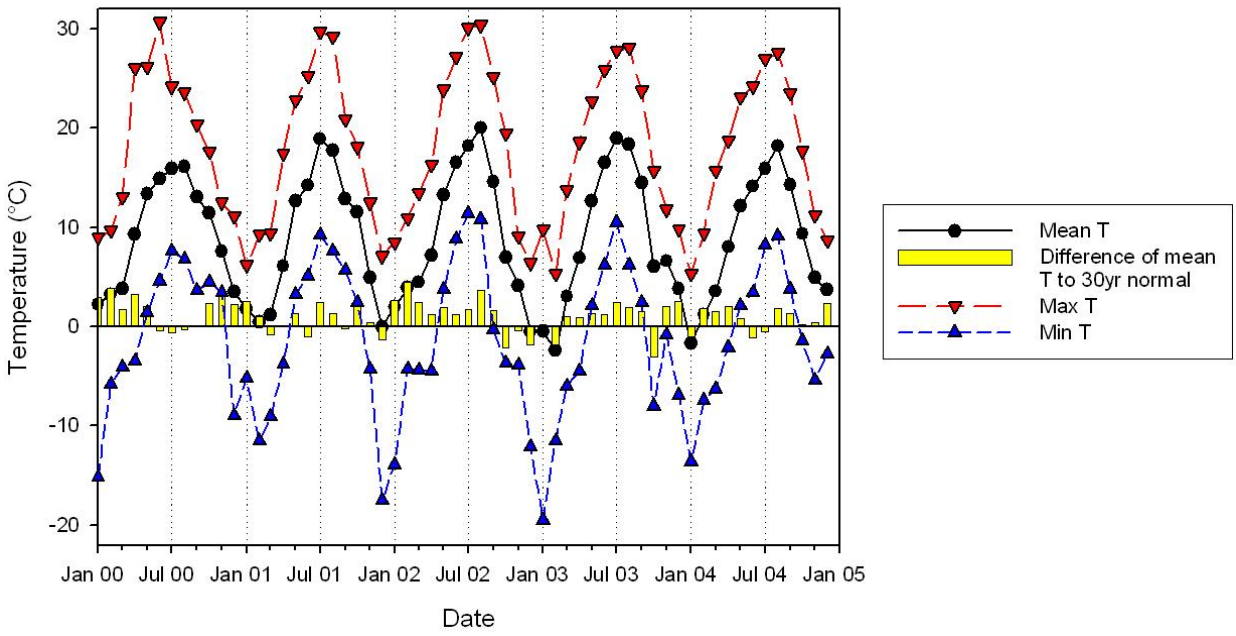


**Fig. 7:** Monthly temperature values for meteorological observation station Malmö A 2000-2004.

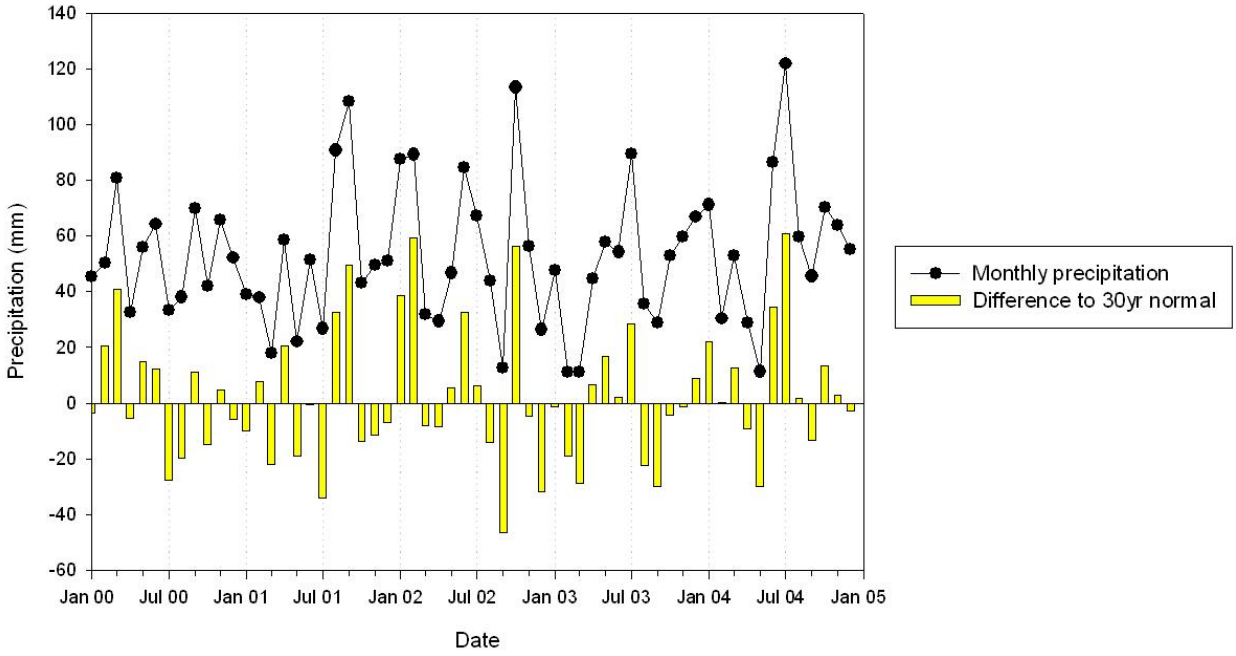


**Fig. 8:** Monthly precipitation values for meteorological observation station Malmö A 2000-2004

# CHAPTER 4

## Adult survival of different genotypes in all-hybrid populations of hybridogenetic water frogs

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## Abstract

The hybridogenetic frog *Rana esculenta* is formed by interspecific matings between *R. lessonae* and *R. ridibunda* and therefore possesses the genomes of both species (LR-genome). Being a sexual parasite, *R. esculenta* usually forms populations with one of its parental species. In the northern range of its distribution, however, *R. esculenta* also lives in all-hybrid populations that are devoid of any parental genotypes, but consist of triploid genotypes (LLR and LRR) besides the usual diploid LR animals. All genotypes are present in every pond, but relative genotype compositions vary between populations. A possible factor for the differences in population composition may be differential survival of the different hybrid genotypes, perhaps originating from varying dosage effects.

In this study, we estimated survival probabilities of all genotypes in multiple ponds inhabited by all-hybrid genotypes in southern Sweden and we assessed which factors best explain variation in survival. Patterns of survival among different genotypes were highly variable among ponds and model selection yielded not one single candidate model for adult survival that was best supported by the data. We show that all frogs, regardless of genotype or sex, have very similar biweekly survival rates of around 90% with usually lower survival probabilities during the breeding season than in between breeding seasons. Differential survival of genotypes in the adult stage is therefore not a mechanism that affects the composition of all-hybrid populations.

*Keywords*: all-hybrid populations, hybridogenesis, *Rana esculenta*, triploid, survival, mark-recapture, model selection

## Introduction

Understanding demographic mechanisms at the population level has become increasingly important in organisms with a complex life-cycle, such as pond-breeding amphibians (Biek et al. 2002, Beebee 2005, Scherer et al. 2005, Schmidt et al. 2005). Amphibians exhibit a complex life cycle with aquatic egg and larval stages, a transformation period called metamorphosis, followed by terrestrial juvenile and adult stages. Such multi-stage development allows population regulation to occur at one or multiple stages (Hellriegel and Reyer 2000). Most studies have focused on the larval stages influencing population dynamics (Wilbur and Collins 1973, Wilbur 1980, 1997, Semlitsch 2003). From these studies, it seems that survival in the aquatic life stage is a main factor regulating population dynamics. More recent studies have included terrestrial juvenile stages into their analyses and found population regulation effects also at such stages (Biek et al. 2002, Vonesh and De la Cruz 2002, Schmidt et al. 2005, Chelgren et al. 2006). Finally, population fluctuations may also be governed by variation in adult survival (Holenweg Peter 2001, Frétey et al. 2004, Schmidt et al. 2005) in addition to other factors operating during the adult stage, such as fecundity, sex ratio or dispersal. For amphibians, there is plenty of data on larval survival rates under experimental conditions (e.g., Wilbur 1997), but data on survival under natural conditions is scarce for both larvae and adults because it is difficult to measure (Govindarajulu and Anholt 2006). Recent advances in marking techniques and new analyses of capture-recapture data now allow a powerful approach to estimate survival rates under natural conditions. These new capture-recapture analyses yield more reliable results than the commonly reported return rates (e.g., Jehle et al. 1995, Martin et al. 1995, Hels 2002) because they additionally account for the fact that individuals are not always detected. They also allow to test which factors affect survival (Lebreton et al. 1992). In spite of these advantages, the new survival estimation methods have not been used too often by amphibian ecologists so far (Schmidt et al. 2002).

Understanding demography and population regulation is most interesting in systems where multiple species or genotypes share a habitat because different life histories and

population regulation mechanisms may contribute to coexistence (Vrijenhoek et al. 1992, Vrijenhoek and Pfeiler 1997, Declerck and De Meester 2003). Here, we investigate variation in adult survival of three sympatric hybrid genotypes because it may contribute to the maintenance of all-hybrid populations of hybridogenetic water frogs.

The European hybridogenetic water frog *Rana esculenta* originates from matings between the two parental water frog species *R. lessonae* and *R. ridibunda*. Hybidogenesis is a special reproductive mode, where one of the two parental genomes is excluded by the hybrid prior to meiosis, while the other is passed on clonally (Schultz 1969). To produce hybrid offspring again, *R. esculenta* is therefore typically forced into syntopy and mating with the respective parental species whose genome was excluded (Graf and Polls Pelaz 1989). In Western Europe, associations between *R. lessonae* and *R. esculenta* prevail, forming so-called LE-systems. In eastern Europe, *R. esculenta* is more often found with *R. ridibunda* in RE-systems (see Plötner 2005 for geographic distributions of the different population systems). A striking feature of these mixed populations is that they can differ markedly in their composition (Blankenhorn 1977, Holenweg Peter et al. 2002). E.g., proportions of parental and hybrid individuals may range from 5% *R. lessonae* and 95% *R. esculenta* to the reverse ratio (Berger 1988). The factors that were found to account for these differences included biotic and abiotic factors in the larval and adult habitat, as well as physiological, behavioural and life-history differences between hybrids and the parental species (Semlitsch 1993a, b, Hellriegel and Reyer 2000, Negovetic et al. 2001, Reyer et al. 2003, Wälti and Reyer 2007). Holenweg Peter (2001) showed in a Swiss population of water frogs that adult survival rates were higher in the parental species *R. lessonae* than in the hybrid *R. esculenta* and that this difference could compensate for the initial reproductive advantage of the hybrid and stabilize the system. Such an adult survival advantage of *R. lessonae* could not be confirmed in a study on another Swiss population (Anholt et al. 2003). Hence, differences in adult survival may vary with ecological conditions and thus be one of the key factors for differences in population structure.

A special type of mixed water frog populations are the all-hybrid ("pure hybrid") populations found mainly at the northern distribution edge (Ebendal 1979, Fog 1994, Plötner 2005). They consist of diploid (LR), two types of triploid (LLR, LRR) and even tetraploid animals (Ebendal and Uzzell 1982, Günther 1990, Berger and Berger 1994, Rybacki and Berger 2001, Jakob et al., chapter 2 in this publication). Although some hybrid x hybrid matings produce offspring with parental genotypes (Jakob and Arioli, chapter 5 in this publication, Arioli and Jakob, chapter 6 in this publication, Arioli 2007), these are absent from the adult populations because the respective larvae are not viable, probably due to the accumulation of deleterious mutations (Vorburger 2001). The role of providing the premeiotically excluded genome is taken over by triploid hybrids (see Jakob and Arioli, chapter 5 in this publication, Arioli and Jakob, chapter 6 in this publication). Thus, the hybrid has become reproductively independent from the parental species in these populations (Som and Reyer 2006). Earlier investigations have shown that sex ratios are skewed in the triploid hybrids: LRR come almost exclusively as females, while the male:female ratio in LLR is 2:1. Moreover, all-hybrid ponds differ markedly in the proportions of the three types (Jakob et al., chapter 2 in this publication).

The aim of this study was to investigate potential differences in survival rate between genotypes and whether these could explain the different population compositions in the area, respectively the skewed sex ratios of triploid genotypes.

## Methods

The study area was located in Skåne (Scania), which forms the southernmost part of Sweden. Twelve ponds were sampled in the years 2002 to 2004 at least twice during the breeding season (May to July). In the first year, 20-30 adult animals were caught at each occasion. In 2003 and 2004, juvenile animals were also included in the sample and sample sizes were extended to up to over 50 individuals, depending on numbers present at the pond. Animals were individually marked upon first capture with a RFID PIT tag (Trovan ID101, Trovan Ltd., UK), which was implanted subcutaneously. Because

the tip of two hind toes were cut for tissue sampling, animals with lost tags could also be recognized as recaptured, but not individually identified as there was no clipping pattern. Genotype identification was performed with a combination of microsatellite analyses of tissue samples and flow cytometry based on a blood sample. This combination of methods allows for a reliable identification of diploid, triploid and tetraploid animals, as well as mosaic genotypes (Jakob and Arioli, chapter 1 in this publication). Triploid animals with repeatedly contradictory results from the analyses were classified as "mixed". Sex determination of adults is straightforward due to external male characteristics like vocal sacs or swollen thumb pads (Brodmann-Kron and Grossenbacher 1994). Most juvenile frogs, however, do not show external sexual dimorphism yet. For this study, juvenile frogs that were not recaptured at the adult stage were arbitrarily assigned to a sex, according to the present, pond-specific sex distribution in adults of the same genotype. A total of 20 possible categories was created this way, arising from five possible genotypic classifications (LR, LLR, LRR, mosaic, and mixed) times two sexes (males and females) times two "age" categories, i.e., whether the frog was caught first as an adult or as a juvenile. For every pond, only categories consisting of more than one entry were used for the analysis to avoid computational problems.

Animals were released at the same pond usually within 24 hours after capture. If the same pond was sampled again before the release of the animals from the previous sampling, the two capture events were pooled. Animals that were permanently removed from the population for crossing experiments or behavioral studies were coded accordingly in the data sets.

We used the program U-CARE 2.2.5 for Windows for goodness-of-fit testing. U-CARE also delivers tests for specific forms of deviation from the fully time-dependent Cormack-Jolly-Seber (CJS) model, such as transience or trap-dependence (trap-shyness or trap-happiness) (Choquet et al. 2005). Ponds were *a priori* analyzed independently because (i) data structure was similar but not equal (e.g., different time intervals between the two capture events within a year or some genotypes never caught in some

ponds) and because (ii) ponds were far apart and we expected no dispersal. If the data were insufficient for running the tests for the full model (e.g., because of too many subgroups or too sparse data), we instead tested a fully time-dependent CJS model.

Model building and selection was performed with the program MARK (White and Burnham 1999). The full model was described as φ(gt+a+s+t) p(gt+a+s+t), where φ denotes survival probability, p recapture probability, gt the genotype, a the age class at first capture (juvenile or adult, modeled similarly to an effect of transients, see Schmidt et al. 2002), s the sex of the animal and t time-dependence. Frogs that were captured as juveniles were coded as juveniles for the first year and promoted to the adult category in the next year. Survival estimates were computed for intervals of two weeks. In a first step, we selected a best model for capture probabilities. To do so, we set φ as constant and searched for the most parsimonious model for p for each pond. We subsequently used this model for detection probability for all models describing different effects on survival. Whenever there were 2 models for encounter rate with a similar fit, both were used in the process of selecting a best model for survival. Because we captured and recaptured relatively few juveniles and because we were primarily interested in sex- and genotype-specific survival of adults, we set juvenile survival equal across all capture events and genotype-sex combinations.

We defined a small set of candidate models that are related to our biological questions. All models are simplifications of the most complex model φ(gt+a+s+t) p(gt+a+s+t) and are shown in Table 1. To keep the set of candidate models simple, time dependence was only included in the model selection process as φ(gt+t), and only if φ(gt) turned out to be the most parsimonious model for survival, because temporal variation in survival could give balancing selection and maintain all genotypes in the population. Besides simple time-dependence, we incorporated a rough seasonality effect (differential winter and summer survival, ws) into our models by treating within-season survival probabilities as summer survival and between-season survival probabilities as winter survival. Model selection was performed using $AIC_c$ (small-sample-size corrected Akaike information criterion) (Burnham and Anderson 2002). Models with a $\Delta AIC_c < 2$ were

considered equally competitive. Parameter estimates were derived through model averaging based on Akaike weights (Lebreton et al. 1992, Burnham and Anderson 2002). Only models with an Akaike weight of >0.01 were used for model averaging.

## Results

In each of the three study years, we conducted at least two capture events per pond; in pond 089 we sampled three times each in 2002 and 2003. In 2002, 2003 and 2004, we captured and marked 866, 1045 and 837 animals for the first time, respectively. Additionally, we recaptured 107 individuals in 2002, 171 in 2003 and 242 in 2004.

GOF testing of data from all ponds revealed that the full model fit the data in all cases adequately (all p>0.08). The GOF test suggested that the assumptions of the CJS model (no transients, no trap-dependence, no overdispersion) were met.

The best model for encounter probability p varied among ponds. The model with constant recapture probability $\varphi(.)$ p(.) was only selected in pond 032A. Model $\varphi(.)$ p(gt+s+t) exhibited the lowest $AIC_c$ values for ponds 001, 011, 032, 111, 126 and 138. This means that encounter rates in these ponds varied with genotype, sex and time. For pond 138, model $\varphi(.)$ p(gt+t) without a sex effect was considered equally competitive ($\Delta AIC_c < 2$) and therefore also entered model selection. This model was also selected for ponds 014 and 108. Age at first capture was only included in ponds 089, 102 and 134 in the encounter probability model $\varphi(.)$ p(gt+s+a+t).

In terms of survival, there was also was no single candidate model that was best supported by the data in all ponds. When investigating the most parsimonious model among those that underwent the model selection process (see Table 1), seasonality (ws) had a large influence on survival in most ponds (see Tables 1-15), i.e., 011, 014, 032, 089, 102, 108, 126, 134 and 138, either directly or in combination with other factors. Further, age at first capture influenced survival in many ponds (001, 014, 032A, 102, 108, 111), except, of course, in ponds 032, 126 and 138, where no juveniles were recaptured at all. Also, sex (ponds 001, 011, 032, 032A, 102, and 138) and genotype (001, 014, 089,

111, 126, 134) had some influence on survival, although mostly only in combination with other factors. Across all ponds, there was no single factor that consistently influenced survival (see Table 2).

Survival estimates (on a biweekly basis) derived through model averaging are high throughout all ponds and all categories (shown in Tables 16, 17). They consistently ranged around 90%; lower values had large standard errors. In general, biweekly survival within the breeding season (summer) was lower than biweekly survival between years (winter). However, in pond 102 this pattern was reversed (Fig. 1). Male and female biweekly survival was about equal over all ponds. In the two ponds where sex had the strongest influence on survival, patterns were opposite: In pond 001, males survived worse than females, contrary to pond 138, where females survived not as well as males (Fig. 2). Over all ponds, biweekly survival probabilities for genotypes were very similar, with a slight survival advantage for LRR animals compared to the other main genotypes (LRR: 92.8%, Mosaic: 93.8%, Mixed: 88.1% LLR: 86.7% and LR: 85.7%). The only exception was pond 089, where survival estimate patterns varied between genotypes (Fig. 3).

Additionally to the biweekly survival estimates derived from the parameter estimation of the model averaging, we also calculated yearly survival rates for the different groups. Yearly survival was composed as follows: (summer survival) $^{(\text{number of time intervals})}$ * (winter survival) $^{(\text{number of time intervals})}$. Table 18 shows that small differences in biweekly survival estimates can result in large differences in yearly survival estimates. For the period of 2002-2003, yearly survival estimates averaged over all adult main genotypes ranged from 0.1405 (pond 032A) to 0.5978 (pond 138). For the period of 2003-2004, they ranged from 0.1209 (pond 102) to 0.6066 (pond 138).

## Discussion

The investigated ponds are well separated from each other and amphibians are also thought to exhibit high site fidelity and limited dispersal capability (Beebee 2005), although exceptions are known from the literature (see review of Smith and Green 2005). However, the few observed dispersers between the closest ponds in our study

(011 and 014, 0.8km; 032, 032A and 126 (0.8 – 1.5km) were rare enough to not influence the validity of our single-state (pond-by-pond) approach.

All frogs, regardless of their sex or genotype, had very similar biweekly survival rates in all ponds. Biweekly survival rate was rather high, both for summer and winter survival; in most of the ponds it was around ninety percent. Similar, if somewhat lower, biweekly survival rates in *Rana esculenta* - and also its parental species *Rana lessonae* - have been found in earlier studies. Also, our estimated annual survival rates of around 30% are comparable to these studies (Holenweg Peter 2001, Anholt et al. 2003). Other amphibian studies (Berven 1990, Sjögren Gulve 1991) have found much lower survival rates than we detected in some of our ponds (e.g., 001, 126, 138). However, this might be due to methodological rather than biological reasons (Martin et al. 1995). Most of the earlier studies did not distinguish between survival and recapture probabilities and since recapture probabilities are far from 1, they usually underestimated survival rates (Schmidt et al. 2002). On the other hand, the comparably low yearly survival estimates for other ponds in our study, such as 011, 014, 102 and 108, might be due to unaccounted emigration to nearby ponds that were not sampled. Pond 032A seems to be a special case, because no breeding at that pond was observed over the study period (Jakob and Arioli, chapter 3 in this publication). Frogs caught at 032A might therefore be considered as migrants en route to another pond.

Most studies also ignore seasonal differences in survival. However, seasonal variation in survival is likely. For example, high activity during the breeding season may lead to elevated mortality compared to the non-breeding season or cold winter weather may kill many frogs. The estimated lower summer than winter survival may be due to higher predation or elevated mortality due to the strenuous breeding activity. Alternatively, it may simply be an artefact of non-detected dispersal between years or even permanent emigration from the pond. From climatic data we know that winters were benign during the study period, which may have contributed to the higher winter survival rates (Holenweg Peter 2001, Anholt et al. 2003). Only long-term studies can reveal if these

survival rates are typical for Southern Sweden or if the climatic conditions were especially favourable in the investigated time period (Altwegg et al. 2006).

Different factors best explained variation in survival among individuals in different ponds. Consequently, across all ponds neither sex nor genotype had a strong effect on survival of adult hybrid frogs. Although in some ponds models with genotype (or sex) influence were ranked high, no clear corresponding patterns between genotype- or sex-specific survival and relative genotype abundance were found. E.g., for pond 126, LLR was the least abundant genotype with the lowest survival probability. But LR, although with the highest survival probability, was only the second-to-most abundant genotype in 2002 and 2003 (Jakob et al., chapter 2 in this publication). Likewise, pond 001 showed a clear survival advantage for females, but many more males than females were present in our samples. Using skeletochronology on frogs from our study ponds, Embrechts (2005) showed that the age structure across ponds, is not different for the three main genotypes LR, LLR and LRR. There was, however, an interaction between genotype and pond type on the mean age. This result is in accordance to our findings that sexes or genotypes do not differ in their overall survival, but survival of frogs of different genotypes or sex varied among the ponds.

Such variation among ponds (or populations) may also explain the seemingly contradictory results on survival in mixed *R. esculenta* / *R. lessonae* populations from Switzerland (Holenweg Peter 2001, Anholt et al. 2003). It is known from the mixed systems that hybrid and parental taxa have quite different habitat requirements (Holenweg Peter et al. 2002, Plénet et al. 2005) and also exhibit varying life-history traits such as annual and life-time fecundity and larval and adult survival (Wälti and Reyer 2007). Therefore, we expected habitat-specific performance of L and R genotypes and different survival in all-hybrid populations, due to different dosage effects in LR, LLR and LRR. But such differences were not detected. Our results from multiple populations show that differential survival of genotypes in the adult stage is unlikely to affect population regulation and the composition and stability of the all-hybrid system. This is consistent with the idea that changes in the genotypic composition, dynamics and

persistence of the all-hybrid populations may be driven mainly by stochastic events (Som and Reyer 2006, Jakob and Arioli, chapter 3 in this publication).

Finally, we could not explain the skewed sex ratios of triploid genotypes (LLR towards males, LRR towards females) by differential survival rates through both sex and genotype (gt+s). E.g., the rarity of LRR males is not due to lower survival rates compared to LRR females. Rather, these skews seem to be produced by some male determining factors linked to the L-genome, so that the production of LRR males is rare in the first place. However, sex determination in *R. esculenta* is more complex than in a simple XY-system, which instead of all-female LRR individuals leaves room for the LRR males (and LLR females, respectively) found in our samples (see also Berger et al. 1988).

## Acknowledgements

We were granted access to a FACSCalibur flow cytometric device by the group of Prof. Rolf Zinkernagel at University Hospital Zürich. We also thank Eva Niederer at the Institute of Biomedical Engineering at ETH Zürich and Bo Baldetorp of Jubileum Institutet at Lund University for their advice. Sandra Röthlisberger supported us in PCR procedures. Our supervisor Heinz-Ulrich Reyer commented on the manuscript.

The work was funded by the Swiss National Science Foundation, grant No. 3100-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-18591-02, 522-9286-03, 522-6571-04). BRS was supported by the "Fondation MAVA".

## Author contributions

CJ and MA contributed equally to this work. Both authors carried out all field- and lab work together. CJ performed statistical analyses and wrote the paper. All authors designed the analysis and discussed the results, MA and BRS commented on the manuscript.

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## Tables

**Table 1:** Fitted models for survival and their biological consequences, sorted by their mean Akaike weights. φ=survival probability, gt=genotype, s=sex, a=age at first capture, ws=seasonality, t=time. Both φ(gt+t) and φ(gt+s+a+t) were not used in the model averaging process and therefore, no Akaike weight was computed.

| Model notation | Biological meaning | Mean Akaike weight |
|---|---|---|
| φ(s+ws) | Differential survival of sexes depending on season (summer/winter) | 0.150 |
| φ(a) | Survival differing between juveniles and adults | 0.147 |
| φ(gt+ws) | Differential survival of genotypes depending on season (summer/winter) | 0.133 |
| φ(.) | Constant survival | 0.117 |
| φ(ws) | Survival differing between summer (breeding season) and winter | 0.115 |
| φ(gt+a) | Differential survival of genotypes depending on age class (juvenile or adult). For instance, if certain genotypes grow faster than others during their juvenile stage and thus escape predation, but survival in the adult stage is determined otherwise. | 0.109 |
| φ(s) | Survival sex-dependent (e.g. due to higher exposure risks of males during mating season or higher physiological investment of females due to spawning)) | 0.076 |
| φ(gt) | Genotype-dependent survival | 0.061 |
| φ(s+a) | Differential survival of sexes depending on age class (juvenile or adult). | 0.061 |
| φ(t) | Full time dependency | 0.037 |
| φ(gt+s) | Differential survival of genotypes depending on sex | 0.036 |
| φ(gt+t) | Differential survival of genotypes over the sampling period | - |
| φ(gt+s+a+t) | Full model | - |

**Table 2:** Cumulative Akaike weights of single model factors (per pond and mean over all ponds). All Akaike weights from a model containing the specific factor were added. Therefore, the sum of Akaike weights does not add up to 1. Highest values are printed bold.

| Factor | 001 | 011 | 014 | 032 | 032A | 089 | 102 | 108 | 111 | 126 | 134 | 138 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| s | **0.487** | 0.447 | 0.137 | 0.608 | 0.230 | 0.176 | 0.204 | 0.122 | 0.084 | 0.137 | 0.116 | 0.914 | 0.305 |
| ws | 0.142 | **0.456** | 0.206 | **0.878** | 0.088 | **0.954** | **0.466** | 0.342 | 0.018 | 0.393 | **0.396** | **0.414** | **0.396** |
| gt | 0.293 | 0.099 | 0.389 | 0.157 | 0.152 | 0.760 | 0.066 | 0.055 | **0.687** | **0.539** | 0.263 | 0.226 | 0.307 |
| a | 0.293 | 0.104 | **0.605** | 0 | **0.333** | 0.013 | 0.229 | **0.350** | 0.732 | 0 | 0.166 | 0 | 0.235 |
| t | 0.122 | 0.043 | 0.056 | 0.002 | 0.326 | 0.013 | 0.065 | 0.095 | 0.025 | 0 | 0.012 | 0 | 0.063 |
| . | 0.093 | 0.229 | 0.065 | 0.102 | 0.118 | 0 | 0.193 | 0.133 | 0.046 | 0.168 | 0.350 | 0.026 | 0.127 |

**Table 3:** Model selection results for pond 001. φ()=survival probability, p()=recapture probability, gt=genotype, s=sex, a=age at first capture, ws= seasonality, t=time, (.)=constant. AICc=small-sample-size corrected Akaike information criterion. The model with the lowest AICc value is the most parsimonious of the fitted models and is selected. ΔAICc is the difference in AICc to the selected model. Akaike weights indicate the relative support of a model compared to the other models. Par is the number of parameters that could be calculated by MARK. Deviance is the difference in -2log(Likelihood) of the current model and -2log(Likelihood) of the saturated (fully parametrized) model. Only models with an Akaike weight of >0.01 were used for model averaging and are listed here. As references, also the models for constant survival and recapture probabilities (φ(.) p(.)) and the CJS model φ(t) p(t) were added to the table, but not included in model averaging (except for pond 032A).

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(s) p(gt+s+t) | 161.835 | 0 | 0.239 | 11 | 61.209 |
| φ(s+a) p(gt+s+t) | 163.057 | 1.22 | 0.130 | 12 | 60.012 |
| φ(gt+a) p(gt+s+t) | 163.127 | 1.29 | 0.125 | 13 | 57.624 |
| φ(t) p(gt+s+t)) | 163.175 | 1.34 | 0.122 | 12 | 60.131 |
| φ(.) p(gt+s+t) | 163.715 | 1.88 | 0.093 | 11 | 63.089 |
| φ(gt+s) p(gt+s+t) | 164.421 | 2.59 | 0.066 | 13 | 58.978 |
| φ(gt+ws) p(gt+s+t) | 164.659 | 2.82 | 0.058 | 14 | 56.655 |
| φ(s+ws) p(gt+s+t) | 164.895 | 3.06 | 0.052 | 13 | 59.392 |
| φ(gt) p(gt+s+t) | 165.233 | 3.40 | 0.044 | 13 | 59.730 |
| φ(a) p(gt+s+t) | 165.496 | 3.66 | 0.038 | 12 | 62.451 |
| φ(ws) p(gt+s+t) | 165.832 | 4.00 | 0.032 | 12 | 62.788 |
| φ(.) p(.) | 181.531 | 19.70 | 0.000 | 2 | 100.994 |
| φ(t) p(t) | 185.900 | 24.06 | 0.000 | 9 | 89.992 |

**Table 4:** Model selection results for pond 011. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(s+ws) p(gt+s+t) | 179.217 | 0 | 0.289 | 10 | 30.571 |
| φ(.) p(gt+s+t) | 179.679 | 0.46 | 0.229 | 10 | 31.033 |
| φ(ws) p(gt+s+t) | 180.524 | 1.31 | 0.150 | 10 | 31.879 |
| φ(s) p(gt+s+t) | 181.835 | 2.62 | 0.078 | 11 | 30.956 |
| φ(a) p(gt+s+t) | 181.846 | 2.63 | 0.078 | 11 | 30.966 |
| φ(gt+s) p(gt+s+t) | 182.586 | 3.37 | 0.054 | 12 | 29.449 |
| φ(t) p(gt+s+t) | 183.020 | 3.80 | 0.043 | 12 | 29.884 |
| φ(gt) p(gt+s+t) | 183.888 | 4.67 | 0.028 | 12 | 30.751 |
| φ(s+a) p(gt+s+t) | 184.051 | 4.83 | 0.026 | 12 | 30.915 |
| φ(gt+ws) p(gt+s+t) | 184.892 | 5.68 | 0.017 | 13 | 29.475 |
| φ(t) p(t) | 208.427 | 29.21 | 0.000 | 7 | 66.348 |
| φ(.) p(.) | 212.638 | 33.42 | 0.000 | 2 | 81.068 |

**Table 5:** Model selection results for pond 014. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(a) p(gt+t) | 137.533 | 0 | 0.308 | 6 | 34.557 |
| φ(gt+a) p(gt+t) | 138.566 | 1.03 | 0.183 | 8 | 31.171 |
| φ(gt+ws) p(gt+t) | 138.729 | 1.20 | 0.169 | 9 | 29.079 |
| φ(s+a) p(gt+t) | 139.517 | 1.98 | 0.114 | 7 | 34.347 |
| φ(.) p(gt+t) | 140.647 | 3.11 | 0.065 | 6 | 37.671 |
| φ(t) p(gt+t) | 140.979 | 3.45 | 0.055 | 8 | 33.584 |
| φ(ws) p(gt+t) | 141.768 | 4.24 | 0.037 | 6 | 38.792 |
| φ(gt) p(gt+t) | 141.786 | 4.25 | 0.037 | 8 | 34.391 |
| φ(s) p(gt+t) | 142.751 | 5.22 | 0.023 | 7 | 37.581 |
| φ(t) p(t) | 148.339 | 10.81 | 0.001 | 6 | 45.363 |
| φ(.) p(.) | 156.946 | 19.41 | 0.000 | 2 | 62.459 |

**Table 6:** Model selection results for pond 032. Parameter explanation and model selection information are as described in the caption for Table 3. For this pond, no juvenile animals could be included in the input file. Therefore, no "age at first capture" parameter was present in model selection.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(s+ws) p(gt+s+t) | 242.023 | 0 | 0.598 | 16 | 42.811 |
| φ(gt+ws) p(gt+s+t) | 244.730 | 2.71 | 0.154 | 17 | 43.122 |
| φ(ws) p(gt+s+t) | 245.130 | 3.11 | 0.126 | 16 | 45.919 |
| φ(.) p(gt+s+t) | 245.592 | 3.57 | 0.100 | 15 | 48.751 |
| φ(s) p(gt+s+t) | 250.117 | 8.09 | 0.010 | 17 | 48.509 |
| φ(gt) p(gt+s+t) | 252.507 | 10.48 | 0.003 | 18 | 48.475 |
| φ(.) p(.) | 253.287 | 11.26 | 0.002 | 2 | 85.036 |
| φ(t) p(t) | 253.518 | 11.49 | 0.002 | 9 | 70.366 |

**Table 7:** Model selection results for pond 032A. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(t) p(t) | 136.947 | 0 | 0.323 | 6 | 31.129 |
| φ(a) p(.) | 138.479 | 1.53 | 0.150 | 2 | 41.240 |
| φ(s+a) p(.) | 138.935 | 1.99 | 0.120 | 3 | 39.601 |
| φ(.) p(.) | 139.330 | 2.38 | 0.098 | 2 | 42.091 |
| φ(s) p(.) | 139.703 | 2.76 | 0.082 | 3 | 40.370 |
| φ(gt+a) p(.) | 140.228 | 3.28 | 0.063 | 4 | 38.767 |
| φ(gt) p(.) | 141.148 | 4.20 | 0.040 | 4 | 39.688 |
| φ(ws) p(.) | 141.281 | 4.33 | 0.037 | 3 | 41.947 |
| φ(gt+ws) p(.) | 141.332 | 4.38 | 0.036 | 6 | 35.514 |
| φ(.) p(t) | 142.467 | 5.52 | 0.020 | 6 | 36.649 |
| φ(s+ws) p(.) | 143.094 | 6.15 | 0.015 | 5 | 39.472 |
| φ(gt+s) p(.) | 143.397 | 6.45 | 0.013 | 6 | 37.579 |
| φ(t) p(.) | 146.374 | 9.43 | 0.003 | 6 | 40.557 |

**Table 8:** Model selection results for pond 089. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(gt+ws) p(gt+s+a+t) | 427.047 | 0 | 0.747 | 27 | 135.431 |
| φ(s+ws) p(gt+s+a+t) | 429.939 | 2.89 | 0.176 | 26 | 140.766 |
| φ(ws) p(gt+s+a+t) | 433.412 | 6.36 | 0.031 | 26 | 144.239 |
| φ(t) p(gt+s+a+t) | 435.118 | 8.07 | 0.013 | 28 | 141.039 |
| φ(gt+a) p(gt+s+a+t) | 435.186 | 8.14 | 0.013 | 27 | 143.570 |
| φ(t) p(t) | 453.145 | 26.10 | 0.000 | 10 | 200.629 |
| φ(.) p(.) | 479.252 | 52.20 | 0.000 | 2 | 243.499 |

**Table 9:** Model selection results for pond 102. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(ws) p(gt+s+a+t) | 123.910 | 0 | 0.316 | 12 | 24.799 |
| φ(.) p(gt+s+a+t) | 124.987 | 1.08 | 0.184 | 12 | 25.877 |
| φ(a) p(gt+s+a+t) | 125.358 | 1.45 | 0.153 | 13 | 23.739 |
| φ(s+ws) p(gt+s+a+t) | 125.589 | 1.68 | 0.136 | 13 | 23.970 |
| φ(t) p(gt+s+a+t) | 127.069 | 3.16 | 0.065 | 14 | 22.896 |
| φ(s+a) p(gt+s+a+t) | 127.546 | 3.64 | 0.051 | 14 | 23.373 |
| φ(gt) p p(gt+s+a+t) | 128.838 | 4.93 | 0.027 | 14 | 24.665 |
| φ(gt+a) p(gt+s+a+t) | 128.967 | 5.06 | 0.025 | 15 | 22.190 |
| φ(s) p(gt+s+a+t) | 129.768 | 5.86 | 0.017 | 14 | 25.595 |
| φ(gt+ws) p(gt+s+a+t) | 130.088 | 6.18 | 0.014 | 15 | 23.312 |
| φ(.) p(.) | 131.124 | 7.21 | 0.009 | 2 | 54.775 |
| φ(t) p(t) | 139.389 | 15.48 | 0.000 | 8 | 49.867 |

**Table 10:** Model selection results for pond 108. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(ws) p(gt+t) | 175.277 | 0 | 0.295 | 9 | 38.554 |
| φ(a) p(gt+t) | 175.279 | 0.00 | 0.294 | 9 | 38.556 |
| φ(.) p(gt+t) | 177.062 | 1.79 | 0.121 | 9 | 40.339 |
| φ(t) p(gt+t) | 177.763 | 2.49 | 0.085 | 11 | 36.452 |
| φ(s+ws) p(gt+t) | 178.940 | 3.66 | 0.047 | 11 | 37.628 |
| φ(s) p(gt+t) | 179.152 | 3.88 | 0.042 | 10 | 40.151 |
| φ(s+a) p(gt+t) | 179.664 | 4.39 | 0.033 | 11 | 38.353 |
| φ(gt) p(gt+t) | 179.744 | 4.47 | 0.032 | 11 | 38.432 |
| φ(gt+a) p(gt+t) | 180.409 | 5.13 | 0.023 | 12 | 36.757 |
| φ(.) p(.) | 181.685 | 6.41 | 0.012 | 2 | 60.092 |
| φ(t) p(t) | 182.020 | 6.74 | 0.010 | 8 | 47.545 |

**Table 11:** Model selection results for pond 111. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(gt+a) p(gt+s+t) | 184.679 | 0 | 0.530 | 12 | 35.426 |
| φ(a) p(gt+s+t) | 187.193 | 2.51 | 0.151 | 12 | 37.940 |
| φ(gt) p(gt+s+t) | 187.338 | 2.66 | 0.140 | 12 | 38.085 |
| φ(s+a) p(gt+s+t) | 189.345 | 4.67 | 0.051 | 13 | 37.725 |
| φ(.) p(gt+s+t) | 189.573 | 4.89 | 0.046 | 12 | 40.320 |
| φ(t) p(gt+s+t) | 190.765 | 6.09 | 0.025 | 15 | 34.310 |
| φ(ws) p(gt+s+t) | 191.392 | 6.71 | 0.018 | 13 | 39.771 |
| φ(gt+s) p(gt+s+t) | 191.501 | 6.82 | 0.017 | 14 | 37.480 |
| φ(s) p(gt+s+t) | 191.650 | 6.97 | 0.016 | 13 | 40.030 |
| φ(.) p(.) | 200.146 | 15.47 | 0.000 | 2 | 72.925 |
| φ(t) p(t) | 202.082 | 17.40 | 0.000 | 8 | 61.990 |

**Table 12:** Model selection results for pond 126. Parameter explanation and model selection information are as described in the caption for Table 3. For this pond, no juvenile animals could be included in the input file. Therefore, no "age at first capture" parameter was present in model selection.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(gt) p(gt+s+t) | 339.885 | 0 | 0.350 | 22 | 63.097 |
| φ(ws) p(gt+s+t) | 341.175 | 1.29 | 0.184 | 21 | 66.846 |
| φ(.) p(gt+s+t) | 341.352 | 1.47 | 0.168 | 21 | 67.022 |
| φ(gt+ws) p(gt+s+t) | 341.512 | 1.63 | 0.155 | 23 | 62.241 |
| φ(s+ws) p(gt+s+t) | 343.615 | 3.73 | 0.054 | 22 | 66.827 |
| φ(s) p(gt+s+t) | 343.809 | 3.92 | 0.049 | 22 | 67.021 |
| φ(gt+s) p(gt+s+t) | 344.553 | 4.67 | 0.034 | 24 | 62.775 |
| φ(t) p(t) | 353.091 | 13.21 | 0.000 | 8 | 108.646 |
| φ(.) p(.) | 354.476 | 14.59 | 0.000 | 2 | 122.644 |

**Table 13:** Model selection results for pond 134. Parameter explanation and model selection information are as described in the caption for Table 3.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(.) p(gt+s+a+t) | 149.701 | 0 | 0.350 | 10 | 30.465 |
| φ(gt+ws) p(gt+s+a+t) | 150.539 | 0.84 | 0.231 | 12 | 26.760 |
| φ(a) p(gt+s+a+t) | 151.417 | 1.72 | 0.149 | 11 | 29.924 |
| φ(ws) p(gt+s+a+t) | 151.947 | 2.25 | 0.114 | 11 | 30.453 |
| φ(s+ws) p(gt+s+a+t) | 153.539 | 3.84 | 0.051 | 12 | 29.761 |
| φ(s) p(gt+s+a+t) | 154.197 | 4.50 | 0.037 | 12 | 30.419 |
| φ(gt) p(gt+s+a+t) | 155.286 | 5.59 | 0.021 | 13 | 29.197 |
| φ(s+a) p(gt+s+a+t) | 155.803 | 6.10 | 0.017 | 13 | 29.714 |
| φ(t) p(gt+s+a+t) | 156.441 | 6.74 | 0.012 | 13 | 30.351 |
| φ(gt+s) p(gt+s+a+t) | 156.691 | 6.99 | 0.011 | 14 | 28.264 |
| φ(t) p(t) | 182.491 | 32.79 | 0.000 | 7 | 69.880 |
| φ(.) p(.) | 185.873 | 36.17 | 0.000 | 2 | 83.823 |

**Table 14:** Model selection results for pond 138. Parameter explanation and model selection informations are as described in the caption for Table 3. For this pond, juvenile animals were only caught during the very last capture event. Therefore, no "age at first capture" parameter was present in model selection.

| Model | AICc | ΔAICc | Akaike weight | Par | Deviance |
|---|---|---|---|---|---|
| φ(s+ws) p(gt+t) | 255.185 | 0 | 0.312 | 9 | 57.545 |
| φ(s) p(gt+t) | 255.324 | 0.14 | 0.291 | 9 | 57.684 |
| φ(gt+s) p(gt+t) | 256.466 | 1.28 | 0.164 | 11 | 54.208 |
| φ(s+ws) p(gt+s+t) | 258.217 | 3.03 | 0.068 | 13 | 51.207 |
| φ(gt+s) p(gt+s+t) | 258.407 | 3.22 | 0.062 | 14 | 48.969 |
| φ(ws) p(gt+s+t) | 260.886 | 5.70 | 0.018 | 13 | 53.876 |
| φ(s) p(gt+s+t) | 261.017 | 5.83 | 0.017 | 14 | 51.579 |
| φ(ws) p(gt+t) | 261.082 | 5.90 | 0.016 | 9 | 63.442 |
| φ(.) p(gt+s+t) | 261.563 | 6.38 | 0.013 | 13 | 54.553 |
| φ(.) p(gt+t) | 261.837 | 6.65 | 0.011 | 9 | 64.197 |
| φ(.) p(.) | 265.369 | 10.18 | 0.002 | 2 | 82.916 |
| φ(t) p(t) | 268.953 | 13.77 | 0.000 | 8 | 73.573 |

**Table 15:** Summary of Akaike weights for different models of recapture probability. Parameter explanation and model selection information are as described in the caption for Table 3. Values printed in bold are highest Akaike weights per pond.

| Pond | 001 | 011 | 014 | 032 | 032A | 089 | 102 | 108 | 111 | 126 | 134 | 138 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Recapture probability P | (gt+s+t) | (gt+s+t) | (gt+t) | (gt+s+t) | (.) | (gt+s+a+t) | (gt+s+a+t) | (gt+t) | (gt+s+t) | (gt+s+t) | (gt+s+a+t) | (gt+s+t)/(gt+t) |
| Model | | | | | | | | | | | | |
| φ(.) | 0.093 | 0.229 | 0.065 | 0.100 | 0.020 | 0.000 | 0.184 | 0.121 | 0.046 | 0.168 | **0.350** | 0.024 |
| φ(t) | 0.122 | 0.043 | 0.055 | 0.001 | 0.003 | 0.013 | 0.065 | 0.085 | 0.025 | 0.005 | 0.012 | 0.009 |
| φ(ws) | 0.032 | 0.150 | 0.037 | 0.126 | 0.037 | 0.031 | **0.316** | **0.295** | 0.018 | 0.184 | 0.114 | 0.034 |
| φ(gt) | 0.044 | 0.028 | 0.037 | 0.003 | 0.040 | 0.002 | 0.027 | 0.032 | 0.140 | **0.350** | 0.021 | 0.004 |
| φ(s) | **0.239** | 0.078 | 0.023 | 0.010 | 0.082 | 0.005 | 0.017 | 0.042 | 0.016 | 0.049 | 0.037 | 0.308 |
| φ(a) | 0.038 | 0.078 | **0.308** | - | 0.150 | 0.001 | 0.153 | 0.294 | 0.151 | - | 0.149 | - |
| φ(gt+ws) | 0.058 | 0.017 | 0.169 | 0.154 | 0.036 | **0.747** | 0.014 | 0.006 | 0.002 | 0.155 | 0.231 | 0.012 |
| φ(gt+t) | - | | - | - | - | - | - | | - | - | - | - |
| φ(gt+s) | 0.066 | 0.054 | 0.006 | 0.002 | 0.013 | 0.004 | 0.002 | 0.000 | 0.017 | 0.034 | 0.011 | 0.226 |
| φ(gt+a) | 0.125 | 0.009 | 0.183 | - | 0.063 | 0.013 | 0.025 | 0.023 | **0.530** | - | 0.007 | - |
| φ(s+ws) | 0.052 | **0.289** | 0.002 | **0.598** | 0.015 | 0.176 | 0.136 | 0.047 | 0.002 | 0.054 | 0.051 | **0.380** |
| φ(s+a) | 0.130 | 0.026 | 0.114 | - | 0.120 | 0.008 | 0.051 | 0.033 | 0.051 | - | 0.017 | - |
| φ(gt+s+a+t) | - | | - | - | - | - | - | | - | - | - | - |
| φ(.) p(.) | 0.000 | 0.000 | 0.001 | 0.002 | 0.098 | 0.000 | 0.009 | 0.012 | 0.000 | 0.000 | 0.000 | 0.002 |
| φ(t) p(t) | 0.000 | 0.000 | 0.000 | 0.002 | **0.323** | 0.000 | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 |

**Table 16:** Biweekly survival probabilities derived from model averaging, +/- standard error. Group names show age at first capture (Adults or Juveniles), genotype and sex (males and females). Estimates in brackets could not reliably be computed, their upper and lower 95% confident limits are near to 0 and 1, respectively. Pond 089 is presented separately in Table 17 due to its different capture history. *Within 2004 survival can not be calculated independently from encounter probability.

| Pond | Group | Within 2002 | 2002-2003 | Within 2003 | 2003-2004 | Within 2004* |
|---|---|---|---|---|---|---|
| 001 | A_LR_M | 0.837 ±0.251 | (0.859 ±7.680) | 0.878 ±0.245 | (0.855 ±7.680) | 0.862 ±0.243 |
| | A_LR_F | 0.921 ±0.117 | (0.929 ±7.677) | 0.961 ±0.064 | (0.925 ±7.677) | 0.945 ±0.075 |
| | J_LR_M | 0.837 ±0.250 | (0.858 ±7.680) | 0.878 ±0.244 | (0.855 ±7.680) | 0.862 ±0.241 |
| | J_LR_F | 0.916 ±0.112 | (0.924 ±7.677) | 0.956 ±0.058 | (0.920 ±7.677) | 0.940 ±0.069 |
| | A_LLR_M | 0.896 ±0.117 | 0.958 ±0.020 | 0.936 ±0.078 | 0.955 ±0.018 | 0.921 ±0.082 |
| | A_LLR_F | 0.922 ±0.114 | 0.972 ±0.019 | 0.963 ±0.056 | 0.969 ±0.019 | 0.947 ±0.069 |
| | J_LLR_M | 0.891 ±0.116 | 0.953 ±0.027 | 0.932 ±0.079 | 0.950 ±0.025 | 0.916 ±0.082 |
| | J_LLR_F | 0.913 ±0.112 | 0.963 ±0.028 | 0.954 ±0.059 | 0.959 ±0.027 | 0.938 ±0.069 |
| | A_LRR_F | 0.942 ±0.111 | 0.981 ±0.017 | 0.983 ±0.029 | 0.978 ±0.020 | 0.967 ±0.055 |
| | J_LRR_F | 0.927 ±0.108 | 0.966 ±0.029 | 0.968 ±0.037 | 0.963 ±0.028 | 0.952 ±0.056 |
| 011 | A_LR_M | 0.863 ±0.156 | 0.945 ±0.014 | 0.892 ±0.089 | 0.945 ±0.012 | 0.883 ±0.095 |
| | A_LR_F | 0.765 ±0.223 | 0.944 ±0.038 | 0.794 ±0.198 | 0.944 ±0.037 | 0.785 ±0.196 |
| | J_LR_M | 0.862 ±0.155 | 0.944 ±0.019 | 0.891 ±0.090 | 0.944 ±0.038 | 0.882 ±0.095 |
| | J_LR_F | 0.764 ±0.223 | 0.944 ±0.039 | 0.793 ±0.198 | 0.944 ±0.038 | 0.784 ±0.196 |
| | A_LLR_M | 0.858 ±0.157 | 0.945 ±0.019 | 0.887 ±0.095 | 0.945 ±0.017 | 0.877 ±0.099 |
| | A_LLR_F | 0.714 ±0.278 | 0.898 ±0.216 | 0.743 ±0.264 | 0.898 ±0.216 | 0.734 ±0.261 |
| | A_LRR_F | 0.764 ±0.229 | 0.952 ±0.037 | 0.793 ±0.205 | 0.952 ±0.036 | 0.784 ±0.203 |
| | J_LRR_F | 0.763 ±0.229 | 0.951 ±0.038 | 0.792 ±0.205 | 0.951 ±0.037 | 0.783 ±0.203 |
| | A_Mix_M | 0.761 ±0.305 | (0.851 ±14.46) | 0.790 ±0.287 | (0.851 ±14.46) | 0.781 ±0.286 |
| | A_Mix_F | 0.667 ±0.317 | (0.855 ±14.46) | 0.696 ±0.309 | (0.855 ±14.46) | 0.687 ±0.305 |
| 014 | A_LR_M | 0.828 ±0.242 | 0.941 ±0.024 | 0.868 ±0.142 | 0.939 ±0.020 | (0.885 ±2.450) |
| | A_LR_F | 0.827 ±0.242 | 0.939 ±0.023 | 0.867 ±0.142 | 0.938 ±0.020 | (0.883 ±2.450) |
| | J_LR_F | 0.869 ±0.261 | 0.981 ±0.029 | 0.909 ±0.163 | 0.980 ±0.028 | (0.925 ±2.451) |
| | A_LLR_M | 0.864 ±0.248 | 0.959 ±0.034 | 0.904 ±0.143 | 0.957 ±0.032 | (0.920 ±2.449) |
| | A_LLR_F | 0.862 ±0.248 | 0.957 ±0.034 | 0.902 ±0.142 | 0.956 ±0.032 | (0.919 ±2.449) |
| | A_LRR_F | 0.888 ±0.220 | 0.924 ±0.032 | 0.928 ±0.073 | 0.923 ±0.028 | (0.945 ±2.446) |
| | J_LRR_F | 0.934 ±0.230 | 0.969 ±0.046 | 0.973 ±0.078 | 0.968 ±0.045 | (0.990 ±2.746) |
| 032 | A_LR_M | 0.705 ±0.126 | 0.977 ±0.016 | 0.705 ±0.126 | 0.977 ±0.016 | 0.705 ±0.324 |
| | A_LR_F | 0.910 ±0.142 | 0.960 ±0.019 | 0.910 ±0143 | 0.960 ±0.019 | 0.910 ±0.331 |
| | A_LLR_M | 0.721 ±0.139 | 0.979 ±0.017 | 0.721 ±0.139 | 0.979 ±0.017 | 0.721 ±0.329 |
| | A_LLR_F | 0.926 ±0.132 | 0.962 ±0.022 | 0.926 ±0.132 | 0.961 ±0.022 | 0.926 ±0.326 |
| | A_LRR_F | 0.963 ±0.091 | 0.956 ±0.025 | 0.963 ±0.092 | 0.956 ±0.025 | 0.963 ±0.312 |
| 032A | A_LR_M | 0.591 ±0.427 | (0.893 ±6.56) | 0.911 ±0.146 | (0.901 ±6.56) | 0.912 ±0.144 |
| | A_LR_F | 0.614 ±0.430 | 0.924 ±0.021 | 0.934 ±0.097 | 0.933 ±0.024 | 0.935 ±0.094 |
| | J_LR_M | (0.294 ±2.536) | (0.596 ±7.033 | (0.614±2.541) | (0.604 ±7.033) | (0.615 ±2.541) |
| | J_LR_F | (0.307 ±2.537) | (0.617 ±2.538) | (0.627 ±2.541) | (0.626 ±2.539) | (0.628 ±2.541) |
| | A_LLR_M | 0.605 ±0.422 | (0.875 ±10.58) | 0.924 ±0.093 | (0.883 ±10.58) | 0.925 ±0.090 |
| | A_LLR_F | 0.620 ±0.430 | (0.899 ±8.297) | 0.940 ±0.073 | (0.907 ±8.297) | 0.941 ±0.068 |
| | A_LRR_F | 0.625 ±0.434 | 0.922 ±0.020 | 0.945 ±0.074 | 0.931 ±0.024 | 0.945 ±0.070 |
| | A_Mix_M | (0.469 ±3.227) | (0.776 ±3.420) | (0.789 ±3.214) | (0.784 ±3.420) | (0.790 ±3.214) |
| 102 | A_LR_M | 0.961 ±0.044 | 0.915 ±0.023 | 0.950 ±0.059 | 0.913 ±0.021 | 0.961 ±0.067 |
| | A_LR_F | 0.963 ±0.042 | 0.920 ±0.020 | 0.952 ±0.058 | 0.919 ±0.018 | 0.963 ±0.065 |
| | J_LR_M | 0.975 ±0.035 | 0.928 ±0.033 | 0.964 ±0.055 | 0.927 ±0.032 | 0.975 ±0.061 |
| | J_LR_F | 0.975 ±0.034 | 0.932 ±0.029 | 0.964 ±0.055 | 0.931 ±0.029 | 0.975 ±0.061 |
| | A_LLR_M | 0.960 ±0.045 | 0.916 ±0.023 | 0.949 ±0.059 | 0.915 ±0.021 | 0.960 ±0.067 |
| | A_LLR_F | 0.962 ±0.043 | 0.921 ±0.019 | 0.951 ±0.058 | 0.920 ±0.017 | 0.962 ±0.066 |
| | J_LLR_M | 0.973 ±0.038 | 0.929 ±0.033 | 0.962 ±0.057 | 0.927 ±0.032 | 0.973 ±0.063 |

| Pond | Group | Within 2002 | 2002-2003 | Within 2003 | 2003-2004 | Within 2004* |
|---|---|---|---|---|---|---|
| 102 cont. | J_LLR_F | 0.973 ±0.038 | 0.933 ±0.029 | 0.962 ±0.057 | 0.932 ±0.029 | 0.973 ±0.063 |
| | A_LRR_F | 0.968 ±0.040 | (0.919 ±4.666) | 0.957 ±0.057 | (0.917 ±4.666) | 0.968 ±0.064 |
| | J_LRR_F | 0.977 ±0.033 | (0.929 ±4.666) | 0.966 ±0.054 | (0.927 ±4.666) | 0.977 ±0.060 |
| | A_Mix_M | 0.964 ±0.043 | (0.915 ±7.464) | 0.953 ±0.059 | (0.910 ±7.464) | 0.964 ±0.066 |
| | A_Mix_F | 0.964 ±0.043 | (0.911 ±6.424) | 0.953 ±0.059 | (0.914 ±6.424) | 0.964 ±0.066 |
| 108 | A_LR_M | 0.850 ±0.268 | 0.935 ±0.020 | 0.841 ±0.132 | 0.934 ±0.017 | 0.864 ±0.734 |
| | A_LR_F | 0.847 ±0.269 | 0.937 ±0.020 | 0.838 ±0.135 | 0.936 ±0.018 | 0.861 ±0.735 |
| | J_LR_M | (0.681 ±71.36) | (0.765 ±71.36) | (0.672 ±71.36) | (0.763 ±71.36) | (0.695 ±71.36) |
| | J_LR_F | (0.678 ±71.36) | (0.766 ±71.36) | (0.669 ±71.36) | (0.765 ±71.36) | (0.692 ±71.36) |
| | A_LLR_M | 0.850 ±0.267 | 0.935 ±0.019 | 0.841 ±0.130 | 0.934 ±0.017 | 0.864 ±0.734 |
| | A_LLR_F | 0.848 ±0.268 | 0.937 ±0.019 | 0.839 ±0.133 | 0.935 ±0.017 | 0.862 ±0.734 |
| | J_LLR_M | (0.681 ±71.36) | (0.765 ±71.36) | (0.672 ±71.36) | (0.763 ±71.36) | (0.695 ±71.36) |
| | J_LLR_F | (0.678 ±71.36) | (0.766 ±71.36) | (0.699 ±71.36) | (0.765 ±71.36) | (0.692 ±71.36) |
| | A_LRR_F | (0.835 ±5.215) | (0.920 ±5.45) | (0.826 ±5.21) | (0.918 ±5.45) | (0.849 ±5.259) |
| | J_LRR_F | (0.669 ±71.36) | (0.758 ±71.36) | (0.660 ±71.36) | (0.757 ±71.36) | (0.683 ±71.36) |
| | A_Mos_F | 0.845 ±0.278 | 0.939 ±0.068 | 0.836 ±0.151 | 0.938 ±0.067 | 0.859 ±0.738 |
| 111 | A_LR_M | 0.941 ±0.035 | 0.942 ±0.026 | 0.937 ±0.041 | 0.941 ±0.025 | 0.941 ±0.035 |
| | A_LR_F | 0.941 ±0.034 | 0.943 ±0.027 | 0.938 ±0.040 | 0.941 ±0.026 | 0.941 ±0.034 |
| | J_LR_M | (0.253 ±2.783) | (0.254 ±2.783) | (0.249 ±2.782) | (0.253 ±2.783) | (0.253 ±2.783) |
| | J_LR_F | (0.253 ±2.783) | (0.254 ±2.783) | (0.249 ±2.782) | (0.253 ±2.783) | (0.253 ±2.783) |
| | A_LLR_M | 0.642 ±0.250 | 0.636 ±0.248 | 0.631 ±0.246 | 0.642 ±0.246 | 0.642 ±0.250 |
| | A_LLR_F | 0.634 ±0.263 | 0.644 ±0.249 | 0.639 ±0.259 | 0.634 ±0.246 | 0.634 ±0.263 |
| | A_LRR_M | 0.968 ±0.027 | 0.969 ±0.017 | 0.964 ±0.037 | 0.968 ±0.018 | 0.968 ±0.027 |
| | A_LRR_F | 0.969 ±0.024 | 0.971 ±0.015 | 0.965 ±0.035 | 0.969 ±0.017 | 0.969 ±0.024 |
| 126 | A_LR_M | 0.993 ±0.017 | 0.985 ±0.020 | 0.993 ±0.019 | 0.985 ±0.020 | 0.993 ±0.166 |
| | A_LR_F | 0.993 ±0.017 | 0.985 ±0.020 | 0.993 ±0.019 | 0.985 ±0.020 | 0.993 ±0.166 |
| | A_LLR_M | 0.972 ±0.035 | 0.956 ±0.034 | 0.972 ±0.036 | 0.956 ±0.034 | 0.972 ±0.169 |
| | A_LLR_F | 0.973 ±0.034 | 0.958 ±0.034 | 0.973 ±0.035 | 0.958 ±0.034 | 0.973 ±0.168 |
| | A_LRR_M | 0.969 ±0.053 | 0.976 ±0.013 | 0.969 ±0.054 | 0.976 ±0.013 | 0.969 ±0.173 |
| | A_LRR_F | 0.969 ±0.053 | 0.976 ±0.012 | 0.969 ±0.054 | 0.976 ±0.012 | 0.969 ±0.173 |
| | A_Mix_M | 0.995 ±0.010 | 0.989 ±0.014 | 0.995 ±0.013 | 0.990 ±0.014 | 0.995 ±0.165 |
| | A_Mix_F | 0.978 ±0.095 | 0.972 ±0.094 | 0.977 ±0.095 | 0.972 ±0.094 | 0.977 ±0.190 |
| | A_Mos_F | 0.995 ±0.010 | 0.990 ±0.013 | 0.995 ±0.013 | 0.990 ±0.013 | 0.995 ±0.165 |
| 134 | A_LR_M | 0.959 ±0.066 | 0.947 ±0.014 | 0.958 ±0.060 | 0.947 ±0.014 | 0.959 ±0.057 |
| | A_LR_F | 0.948 ±0.085 | 0.949 ±0.018 | 0.947 ±0.080 | 0.949 ±0.018 | 0.948 ±0.078 |
| | J_LR_M | 0.962 ±0.066 | 0.950 ±0.017 | 0.962 ±0.060 | 0.950 ±0.017 | 0.963 ±0.056 |
| | J_LR_F | 0.951 ±0.085 | 0.952 ±0.020 | 0.950 ±0.080 | 0.952 ±0.019 | 0.952 ±0.078 |
| | A_LLR_M | 0.886 ±0.137 | 0.950 ±0.026 | 0.885 ±0.134 | 0.950 ±0.026 | 0.886 ±0.133 |
| | A_LLR_F | 0.875 ±0.141 | 0.953 ±0.027 | 0.875 ±0.138 | 0.953 ±0.027 | 0.876 ±0.138 |
| | A_LRR_F | 0.947 ±0.085 | 0.944 ±0.019 | 0.946 ±0.080 | 0.944 ±0.019 | 0.948 ±0.078 |
| | J_LRR_F | 0.948 ±0.040 | 0.948 ±0.021 | 0.947 ±0.029 | 0.948 ±0.021 | 0.948 ±0.022 |
| | A_Mix_M | 0.956 ±0.070 | (0.828 ±4.68) | 0.955 ±0.064 | (0.828 ±4.68) | 0.957 ±0.061 |
| | A_Mix_F | 0.935 ±0.130 | (0.820 ±4.68) | 0.935 ±0.127 | (0.820 ±4.68) | 0.936 ±0.126 |
| 138 | A_LR_M | 0.995 ±0.013 | 0.993 ±0.014 | 0.995 ±0.013 | 0.993 ±0.014 | 0.992 ±0.065 |
| | A_LR_F | 0.951 ±0.089 | 0.936 ±0.082 | 0.951 ±0.089 | 0.936 ±0.082 | 0.946 ±0.108 |
| | J_LR_M | 0.995 ±0.013 | 0.993 ±0.014 | 0.995 ±0.013 | 0.993 ±0.014 | 0.992 ±0.065 |
| | J_LR_F | 0.951 ±0.089 | 0.936 ±0.082 | 0.951 ±0.089 | 0.936 ±0.082 | 0.948 ±0.108 |
| | A_LLR_M | 0.757 ±0.428 | (0.761 ±4.85) | 0.757 ±0.428 | (0.761 ±4.85) | 0.754 ±0.432 |
| | A_LLR_F | 0.747 ±0.423 | (0.738 ±4.85) | 0.747 ±0.423 | (0.738 ±4.85) | 0.744 ±0.426 |
| | A_LRR_M | 0.999 ±0.005 | 0.998 ±0.008 | 0.999 ±0.005 | 0.998 ±0.008 | 0.997 ±0.064 |

**Table 17:** Biweekly survival probabilities for pond 089 derived from model averaging, +/- standard error. Group names show age at first capture (Adults or Juveniles), genotype and sex (males and females). Estimates in brackets could not reliably be computed, their upper and lower 95% confident limits are near to 0 and 1, respectively. *Within 2004 survival can not be calculated independently from encounter probability.

| Pond | Group | Within 2002.1 | Within 2002.2 | 2002-2003 | Within 2003.1 | Within 2003.2 | 2003-2004 | Within 2004* |
|---|---|---|---|---|---|---|---|---|
| 089 | A_LR_M | 0.834 ±0.113 | 0.831 ±0.111 | 0.999 ±0.001 | 0.828 ±0.116 | 0.833 ±0.113 | 0.999 ±0.001 | 0.834 ±0.113 |
| | A_LR_F | 0.799 ±0.082 | 0.797 ±0.079 | 0.999 ±0.007 | 0.794 ±0.085 | 0.799 ±0.082 | 0.999 ±0.007 | 0.799 ±0.082 |
| | J_LR_M | 0.834 ±0.113 | 0.831 ±0.111 | 0.999 ±0.001 | 0.828 ±0.116 | 0.833 ±0.113 | 0.999 ±0.001 | 0.834 ±0.113 |
| | J_LR_F | 0.800 ±0.084 | 0.797 ±0.081 | 0.999 ±0.003 | 0.795 ±0.087 | 0.800 ±0.084 | 0.999 ±0.003 | 0.800 ±0.084 |
| | A_LLR_M | 0.994 ±0.029 | 0.991 ±0.038 | 0.946 ±0.038 | 0.989 ±0.058 | 0.993 ±0.032 | 0.946 ±0.038 | 0.994 ±0.029 |
| | A_LLR_F | 0.945 ±0.145 | 0.942 ±0.146 | 0.930 ±0.126 | 0.939 ±0.152 | 0.944 ±0.145 | 0.930 ±0.126 | 0.945 ±0.145 |
| | A_LRR_M | 0.858 ±0.085 | 0.855 ±0.084 | 0.999 ±0.007 | 0.852 ±0.091 | 0.857 ±0.085 | 0.999 ±0.007 | 0.858 ±0.085 |
| | A_LRR_F | 0.824 ±0.056 | 0.821 ±0.053 | 0.999 ±0.007 | 0.819 ±0.062 | 0.823 ±0.056 | 0.999 ±0.007 | 0.824 ±0.056 |
| | J_LRR_M | 0.858 ±0.086 | 0.856 ±0.085 | 0.999 ±0.002 | 0.853 ±0.092 | 0.858 ±0.086 | 0.999 ±0.002 | 0.858 ±0.086 |
| | J_LRR_F | 0.825 ±0.058 | 0.822 ±0.055 | 0.999 ±0.004 | 0.819 ±0.064 | 0.924 ±0.059 | 0.999 ±0.004 | 0.825 ±0.058 |
| | A_LLRR_M | 0.995 ±0.028 | 0.992 ±0.037 | 0.999 ±0.001 | 0.990 ±0.057 | 0.995 ±0.031 | 0.999 ±0.001 | 0.995 ±0.028 |
| | A_Mix_M | 0.995 ±0.028 | 0.992 ±0.037 | (0.606 ±36.45) | 0.990 ±0.057 | 0.995 ±0.031 | (0.606 ±36.45) | 0.995 ±0.028 |
| | A_Mix_F | 0.961 ±0.077 | 0.959 ±0.080 | (0.606 ±36.45) | 0.956 ±0.091 | 0.961 ±0.078 | (0.606 ±36.45) | 0.961 ±0.077 |

**Table 18:** Estimated yearly survival between the first catching events from season 2002 to 2003 and from season 2003 to 2004, as well as mean biweekly survival over the whole study period. Values printed in italic letters come with a very large standard deviation.

| Pond | Genotype | yearly 02-03 | yearly 03-04 | Mean biweekly survival |
|---|---|---|---|---|
| 001 | A_LR_M | *0.0149* | *0.0216* | *0.8571* |
| | A_LR_F | *0.1323* | *0.1597* | *0.9299* |
| | J_LR_M | *0.0145* | *0.0216* | *0.8567* |
| | J_LR_F | *0.1143* | *0.1395* | *0.9249* |
| | A_LLR_M | 0.2402 | 0.2978 | 0.9479 |
| | A_LLR_F | 0.3761 | 0.4467 | 0.9646 |
| | J_LLR_M | 0.2083 | 0.2619 | 0.9430 |
| | J_LLR_F | 0.2919 | 0.3457 | 0.9552 |
| | A_LRR_F | 0.5065 | 0.5823 | 0.9759 |
| | J_LRR_F | 0.3333 | 0.3957 | 0.9609 |
| 011 | A_LR_M | 0.2220 | 0.2045 | 0.9367 |
| | A_LR_F | 0.1919 | 0.1409 | 0.9214 |
| | J_LR_M | 0.2162 | 0.1991 | 0.9357 |
| | J_LR_F | 0.1916 | 0.1403 | 0.9213 |
| | A_LLR_M | 0.2207 | 0.2010 | 0.9359 |
| | A_LLR_F | 0.0540 | 0.0385 | 0.8745 |
| | A_LRR_F | 0.2346 | 0.1690 | 0.9280 |
| | J_LRR_F | 0.2285 | 0.1645 | 0.9270 |
| | A_Mix_M | *0.0158* | *0.0142* | *0.8415* |
| | A_Mix_F | *0.0155* | *0.0107* | *0.8308* |
| 014 | A_LR_M | 0.1693 | 0.1538 | 0.9292 |
| | A_LR_F | 0.1608 | 0.1495 | 0.9277 |
| | J_LR_F | 0.4858 | 0.4719 | 0.9697 |
| | A_LLR_M | 0.2850 | 0.2688 | 0.9497 |
| | A_LLR_F | 0.2704 | 0.2607 | 0.9482 |
| | A_LRR_F | 0.1280 | 0.1266 | 0.9232 |
| | J_LRR_F | 0.4228 | 0.4360 | 0.9682 |
| 032 | A_LR_M | 0.2100 | 0.2778 | 0.9317 |
| | A_LR_F | 0.3070 | 0.2984 | 0.9526 |
| | A_LLR_M | 0.2350 | 0.3058 | 0.9364 |
| | A_LLR_F | 0.3386 | 0.3172 | 0.9562 |
| | A_LRR_F | 0.3319 | 0.3011 | 0.9570 |
| 032A | A_LR_M | *0.0649* | *0.0746* | 0.8964 |
| | J_LR_M | *0.0000* | *0.0000* | *0.5991* |
| | A_LR_F | 0.1437 | 0.1768 | 0.9272 |
| | J_LR_F | *0.0000* | *0.0000* | *0.6199* |
| | A_LLR_M | *0.0409* | *0.0466* | *0.8810* |
| | A_LLR_F | *0.0767* | *0.0903* | *0.9043* |
| | A_LRR_F | 0.1373 | 0.1699 | 0.9262 |
| | A_Mix_M | *0.0024* | *0.0023* | *0.7789* |
| 089 | A_LR_M | 0.3891 | 0.4682 | 0.9607 |
| | A_LR_F | 0.3154 | 0.3962 | 0.9520 |
| | J_LR_M | 0.3891 | 0.4682 | 0.9607 |
| | J_LR_F | 0.3158 | 0.3982 | 0.9522 |
| | A_LLR_M | 0.2827 | 0.2855 | 0.9557 |
| | A_LLR_F | 0.1508 | 0.1600 | 0.9328 |
| | A_LRR_M | 0.4485 | 0.5246 | 0.9665 |
| | A_LRR_F | 0.3662 | 0.4466 | 0.9582 |
| | J_LRR_M | 0.4506 | 0.5270 | 0.9667 |
| | J_LRR_F | 0.3685 | 0.4482 | 0.9584 |
| | A_LLRR_M | 0.9426 | 0.9540 | 0.9978 |

| Pond | Genotype | yearly 02-03 | yearly 03-04 | Mean biweekly survival |
|---|---|---|---|---|
| 089 | A_MIX_M | *0.0000* | *0.0000* | *0.6737* |
| | A_MIX_F | *0.0000* | *0.0000* | *0.6688* |
| 102 | A_LR_M | 0.1361 | 0.1100 | 0.9217 |
| | A_LR_F | 0.1538 | 0.1281 | 0.9266 |
| | J_LR_M | 0.1884 | 0.1630 | 0.9353 |
| | J_LR_F | 0.2071 | 0.1791 | 0.9386 |
| | A_LLR_M | 0.1393 | 0.1149 | 0.9228 |
| | A_LLR_F | 0.1574 | 0.1306 | 0.9273 |
| | J_LLR_M | 0.1925 | 0.1616 | 0.9354 |
| | J_LLR_F | 0.2116 | 0.1819 | 0.9391 |
| | A_LRR_F | *0.1509* | *0.1247* | *0.9263* |
| | J_LRR_F | *0.1933* | *0.1643* | *0.9361* |
| | A_Mix_M | *0.1240* | *0.1036* | *0.9194* |
| | A_Mix_F | *0.1366* | *0.1141* | *0.9227* |
| 108 | A_LR_M | 0.1812 | 0.1471 | 0.9236 |
| | A_LR_F | 0.1896 | 0.1534 | 0.9249 |
| | J_LR_M | *0.0014* | *0.0009* | *0.7533* |
| | J_LR_F | *0.0015* | *0.0009* | *0.7541* |
| | A_LLR_M | 0.1812 | 0.1471 | 0.9236 |
| | A_LLR_F | 0.1898 | 0.1500 | 0.9246 |
| | J_LLR_M | *0.0014* | *0.0009* | *0.7533* |
| | J_LLR_F | *0.0015* | *0.0009* | *0.7541* |
| | A_LRR_F | *0.1227* | *0.0954* | *0.9082* |
| | J_LRR_F | *0.0011* | *0.0007* | *0.7460* |
| | A_Mos_M | 0.1987 | 0.1604 | 0.9264 |
| 111 | A_LR_M | 0.2381 | 0.1903 | 0.9411 |
| | A_LR_F | 0.2440 | 0.1911 | 0.9416 |
| | J_LR_M | *0.0000* | *0.0000* | *0.2531* |
| | J_LR_F | *0.0000* | *0.0000* | *0.2531* |
| | A_LLR_M | *0.0000* | *0.0000* | *0.6426* |
| | A_LLR_F | *0.0000* | *0.0000* | *0.6346* |
| | A_LRR_M | 0.4692 | 0.4087 | 0.9681 |
| | A_LRR_F | 0.4925 | 0.4203 | 0.9696 |
| 126 | A_LR_M | 0.7121 | 0.6916 | 0.9862 |
| | A_LR_F | 0.7121 | 0.6916 | 0.9862 |
| | A_LLR_M | 0.3612 | 0.3262 | 0.9584 |
| | A_LLR_F | 0.3786 | 0.3434 | 0.9602 |
| | A_LRR_M | 0.5678 | 0.5204 | 0.9749 |
| | A_LRR_F | 0.5678 | 0.5204 | 0.9749 |
| | A_Mix_M | 0.7801 | 0.7818 | 0.9903 |
| | A_Mix_F | 0.5236 | 0.4853 | 0.9728 |
| | A_Mos_F | 0.7976 | 0.7818 | 0.9908 |
| 134 | A_LR_M | 0.2662 | 0.2380 | 0.9489 |
| | A_LR_F | 0.2693 | 0.2418 | 0.9488 |
| | J_LR_M | 0.2880 | 0.2600 | 0.9520 |
| | J_LR_F | 0.2914 | 0.2633 | 0.9518 |
| | A_LLR_M | 0.2250 | 0.2024 | 0.9392 |
| | A_LLR_F | 0.2323 | 0.2110 | 0.9398 |
| | A_LRR_F | 0.2390 | 0.2123 | 0.9445 |
| | J_LRR_F | 0.2632 | 0.2357 | 0.9479 |
| | A_Mix_M | *0.0137* | *0.0094* | *0.8477* |
| | A_Mix_F | *0.0104* | *0.0070* | *0.8378* |
| 138 | A_LR_M | 0.8347 | 0.8381 | 0.9931 |
| | A_LR_F | 0.1820 | 0.1879 | 0.9379 |
| | J_LR_M | 0.8347 | 0.8381 | 0.9931 |

| Pond | Genotype | yearly 02-03 | yearly 03-04 | Mean biweekly survival |
|---|---|---|---|---|
| | J_LR_F | 0.1820 | 0.1879 | 0.9379 |
| | A_LLR_M | *0.0008* | *0.0008* | *0.7602* |
| 138 | A_LLR_F | *0.0004* | *0.0004* | *0.7391* |
| | A_LRR_M | 0.9502 | 0.9521 | 0.9980 |
| | A_LRR_F | 0.4244 | 0.4482 | 0.9694 |

## Figures

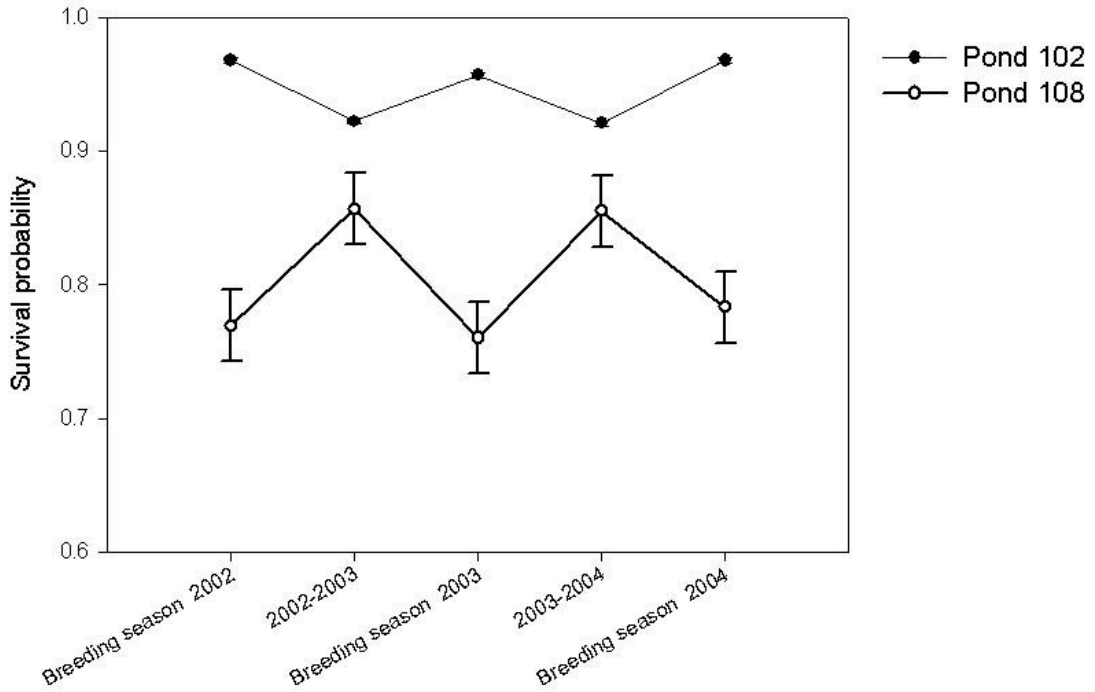


**Fig. 1:** Biweekly survival probabilities during the breeding season (summer survival) and between the years (winter survival). The pattern for pond 108 is the general pattern with lower summer than winter survival. Pond 102 showed an inverse pattern. Error bars show standard error, which was generally large if survival was relatively low.

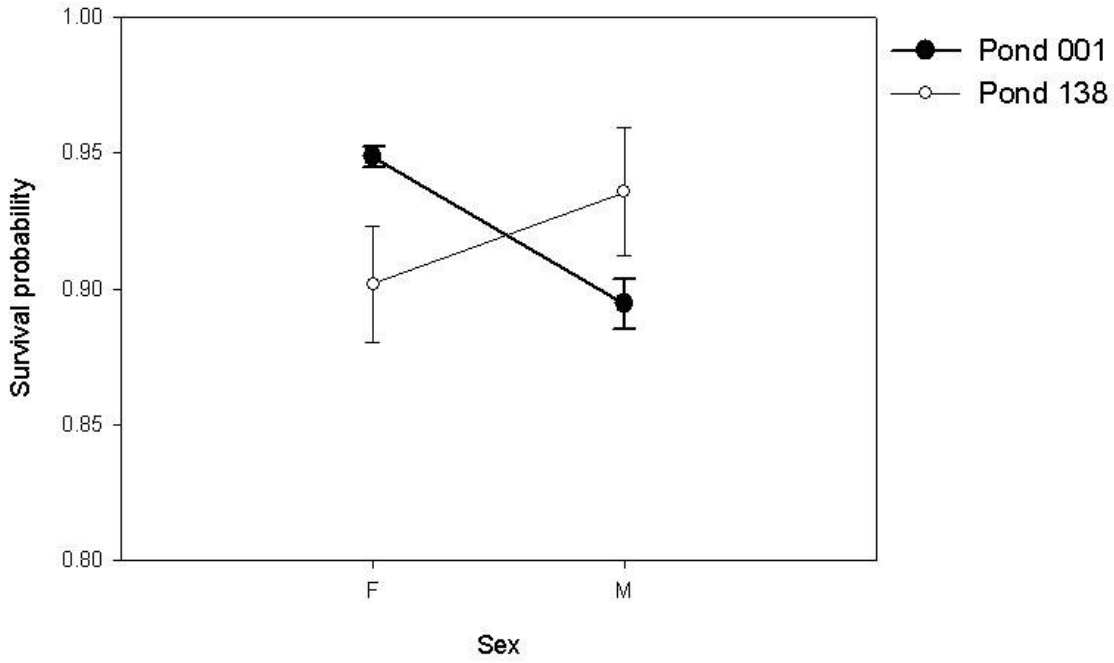


**Fig. 2:** Sex-specific differences in mean survival for females (left data point) and males (right) in ponds 001 and 138. Error bars denote standard error.

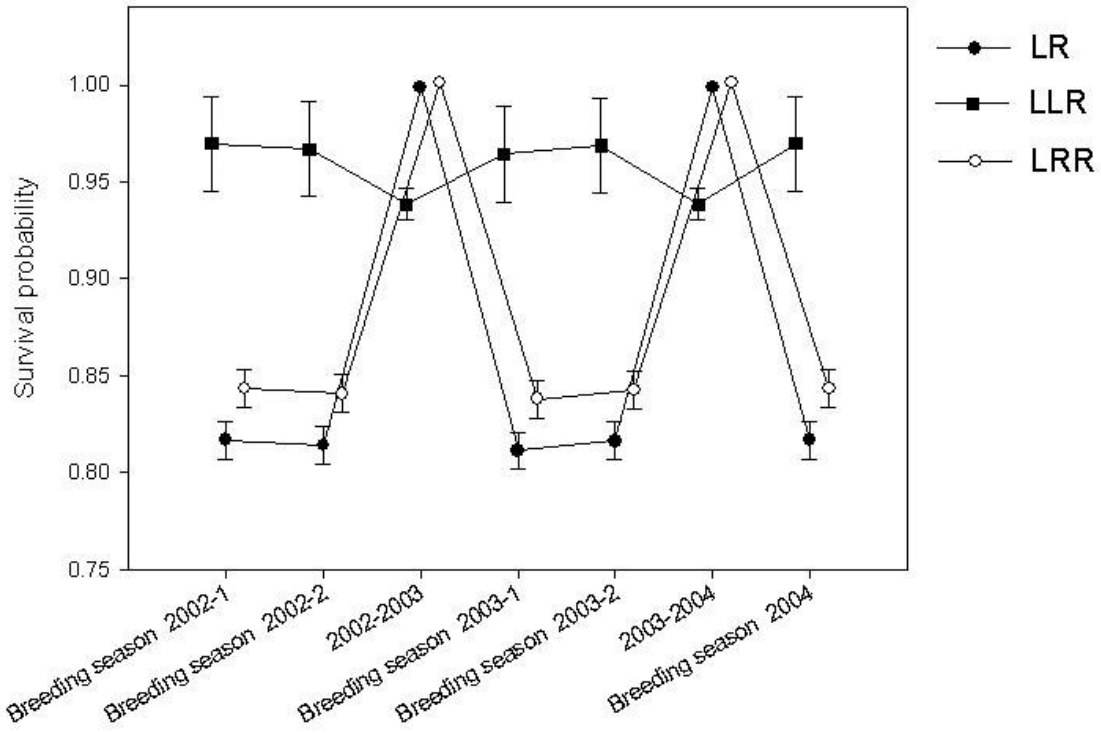


**Fig. 3:** Differences in mean biweekly survival probabilities within and between breeding season and for the three main genotypes in pond 089. This pond was sampled three times during the breeding season in 2002 and 2003. Error bars show standard errors.

# CHAPTER 5

## Occurrence and survival of offspring genotypes in a pure hybrid population of *Rana esculenta*: an experimental approach

CHRISTIAN JAKOB & MARTINA ARIOLI

## Abstract

The evolutionary importance of hybridization has been strongly debated among biologist in the last couple of decades. Most of the formed interspecific hybrids have lower fitness than their ancestors and therefore represent dead ends in evolution. However, some hybrid taxa are surprisingly successful and represent an interesting evolutionary pathway to study. In the Palearctic water frog system several evolutionarily successful hybrid taxa have been formed. In this study, we focus on the edible frog *Rana esculenta*, which originally derived from the mating between the lake frog *R. ridibunda* and the pool frog *R. lessonae*. The bisexual hybrid *R. esculenta* reproduces by hybridogenesis and thus occurs mostly in sympatry with one of its parental species. Special population systems are found at the northern distribution border, i.e., pure hybrid populations consisting of diploid LR frogs as well as triploid LLR and LRR frogs. We investigated if the parental genotypes, which are absent in the adult population, can be formed at all by artificially crossing individuals from hybrid genotypes present in these populations (LR, LLR and LRR). We thereby paid special attention on the pond origin of the mated individuals. Fertilization success and hatchling survival were similar for genotypes in females, but not in males. Our results indicated that diploid LR males were highly variable in their ability to produce offspring, which was mainly due to problems during gametogenesis resulting in simultaneous production of different gamete types. Among the artificially produced offspring were LR, LLR and LRR hybrids, as well as parental genotypes (LL and RR) and tetraploid individuals. These findings

indicate that also diploid sperm was produced. Survival of the parental types among the offspring was lower compared to the hybrid genotypes towards the end of larval development, but not all offspring with parental genotypes died. However, the crossing type, i.e. whether parents came from the same or from different ponds, had no influence neither on fertilization success and hatchling survival nor on later larval survival. This study leads to the conclusion that the total absence of parental genotypes (LL and RR) among adults in nature can not solely be explained by genetic incompatibilities early in larval development, but also requires environmental selection against these genotypes.

*Keywords*: interspecific hybrids, *Rana esculenta*, all-hybrid populations, larval stage, artificial crossings, hybridogenesis, polyploid

## Introduction

Natural hybridization, resulting in the production of offspring between individuals of different species or different populations in the wild, is quite common (reviewed by Arnold 1997, Mallet 2005). Yet the evolutionary importance of hybridization has been strongly debated among biologists in the last couple of decades. Whereas botanist have accepted hybridization as an important evolutionary force in speciation (Stebbins 1959, Grant 1981), zoologist often neglected such an influence and considered it an evolutionary dead end (Dobzhansky 1940, Mayr 1942). Hybrids are usually thought to have lower fitness than the parental species due to endogenous (genetically based) or exogenous (environmentally based) selection acting upon them. Endogenous influences are based on the concurrence of two different genomes, which usually results in difficulties during development and leads to sterility or even death of the newly formed hybrid. Besides such direct genetic problems, there can also be exogenous selection acting on hybrids (Barton 2001). Hybrids often express intermediate behavioral or morphological traits (Grant and Grant 1992, Mavárez et al. 2006, Mendelson and Shaw 2006, Meyer et al. 2006), which may leave them at a disadvantage in the parental habitats. Consequently, an important factor for successful hybrid establishment is the presence of intermediate habitat conditions (Anderson and Stebbins 1954). But in contrast to the assumption that hybrids are unfit relative to their progenitors, Arnold and Hodges (1995) found that occasionally, hybrids are produced that are actually more fit than the parental species and can therefore be of evolutionary importance.

Mostly due to the improvement of molecular techniques, the number of well recognized species today that are actually of hybrid origin is increasing (Seehausen 2004). Many of the hybrid taxa are in fact evolutionary and ecologically very successful (Hedges et al. 1992, Quattro et al. 1992, Alves et al. 2001).

The Western Palearctic water frog complex is a system where hybrids are ecologically as successful as the parental species. At present, ten good species and three hybrid taxa have been described (Plötner 2005). In our study, we concentrate on the best studied of the three hybrid taxa. The edible frog *Rana esculenta* L. (genotype LR) originates from

hybridizations between the lake frog *R. ridibunda* Pallas (genotype RR) and the pool frog *R. lessonae* Camerano (genotype LL). *R. esculenta* was originally considered to be a species, but Berger (1967) discovered through experimental crossings that it is in fact a hybrid with a special mode of reproduction. Tunner (1974) demonstrated that only one unrecombined parental genome is passed on. Later, it was shown that the other half of the genome is excluded from the germline premeiotically (Tunner and Heppich 1981). Thus, *R. esculenta* reproduces by hybridogenesis, a mode of reproduction that had already been described earlier in poecilid fishes by Schultz (1969). With the exception of bisexually reproducing water frogs, hybridogens are unisexual hybrids which, as mentioned above, exclude one of their parental genomes from their germline before meiosis and transmit the other genome clonally to the gametes. A new hybrid is formed by including a "sexual" genome from the parental species whose genome was eliminated previously. This reproduction mode therefore requires that the hybrid lives in sympatry with at least one of its parental species, parasitizing it sexually. Furthermore, it has been shown in mixed *R. lessonae* / *R. esculenta* population systems (so-called "LE-systems") that matings between two hybrids produce inviable offspring (Semlitsch and Reyer 1992, Vorburger 2001) due to the accumulation of deleterious mutations ("Muller's ratchet", Muller 1964).

The hybrid *R. esculenta* possess many intermediate characteristics in relation to the two parental species, such as size, color and vocalization (Plötner 2005). Simultaneously, due to its high degree of heterozygosity, *R. esculenta* is often assumed to exhibit a higher ecological plasticity (Semlitsch and Reyer 1992, Plötner 2005). However, many studies found the hybrid to be intermediate between rather than superior over both parental genotypes, including in physiological requirements (Hotz et al. 1999, Plénet et al. 2000, Negovetic et al. 2001). Such intermediate habitat requirements might facilitate the colonization of different niches within the same habitat of the parent species and could explain the widespread geographical distribution of *R. esculenta*. Not only has the hybrid managed to follow the parents in their distribution, there are even areas where exclusively hybrids are found. In contradiction to the necessary sympatry mentioned above, the hybrid is independent from the sexual progenitors in these areas and mating

can only take place between two hybrids. The formation of these all-hybrid systems without any adult frogs with parental genotypes is not fully understood yet, but the key to their existence is the fact that in these pure hybrid populations, not only diploid animals (LR) are present, but also two types of triploids (LLR and LRR) (Günther et al. 1979, Ebendal and Uzzell 1982, Günther 1983, Graf and Polls Pelaz 1989, Plötner and Klinkhardt 1992, Berger and Berger 1994, Ogielska et al. 2001, Christiansen et al. 2005, Som and Reyer 2006, Jakob et al., chapter 2 in this publication). It is believed that the triploid hybrid forms in pure hybrid populations take over the role of sexual hosts similar to the parental species in mixed LE- and *R. ridibunda* / *R. esculenta* population systems (RE-systems) in Central Europe (see Plötner 2005).

A field study on pure hybrid water frog populations in Southern Sweden has shown that ploidy and genotypic composition varies substantially between populations (Jakob et al., chapter 2 in this publication), which suggests that population systems within this region might differ, similar to systems in mixed populations of hybrids and parental genotypes (LE-, resp. RE- like). In an earlier experiment, the common production of gametes in diploid and triploid *R. esculenta* was determined and it was shown that parental genotypes can be produced in purely hybrid matings and that they may survive until metamorphosis (Arioli 2007). There was a tendency of higher survival for parental genotypes until metamorphosis when parents stemmed from distant ponds. However, this study did not account for the possibility of different population systems and/or hemiclones existing in these ponds. Such differences between ponds can facilitate the production of viable parental genotypes as shown in Switzerland for crossings between different hybrid hemiclones (Vorburger 2001).

To investigate the influence of pond of origin of the parent individuals, and of hypothesized different population systems, we performed a follow-up crossing experiment using artificial fertilization to track the gamete and offspring production in pure hybrid populations in Southern Sweden. We investigated the fertilization and hatching success of the different genotypes and followed the survival success and development of the offspring genotypes during the larval stages.

## Methods

### *Source populations*

Individuals were caught in 3 different ponds in Skåne (Scania), Southern Sweden. Ponds were chosen based on differential population composition, assessed in 2002 and 2003 (Jakob et al., chapter 2 in this publication): Pond 001 was LLR-dominated, 089 LRR-dominated and in pond 011 triploid genotypes were balanced (see Fig. 1). A genotype was considered dominant when adult frogs of this genotype constituted more than 50% of the adult triploid population in 2002 and in 2003. There was a general increase in diploid adult frogs over the two years for most ponds in Skåne (Jakob et al., chapter 2 in this publication), tipping the balance in most ponds dominated by triploids in 2002 towards a diploid majority in 2003. While LLR frogs are slightly biased towards males, LRR frogs are heavily sex-biased towards females, making it difficult to encounter LRR males. All frogs were caught between May 15 and May 19 2004 at night by hand and kept at the field station of the University of Lund at 10°C prior to the crossing. In order to determine their exact genotype we took morphological measurements (snout-vent length, tibia length and length of callus internus), plus a tissue and a blood sample from each individual. Additionally the weight of each individual was measured. Flow cytometry was used to analyze the blood and results, together with the morphological measurements, were used to preliminarily determine the ploidy and genome composition of these frogs (Jakob and Arioli, chapter 1 in this publication). Tissue samples were used in microsatellite analysis to subsequently confirm genotype determination.

### *Samples and crossing design*

From each of the three ponds, we used 3 males and 3 females of each available genotype for the crossing. The only exception was pond 011, where we could only get one LLR female and one supposed LLR female turned out to be an LR after final genotype analyses. This resulted in 4 females for the genotype LR in pond 011 (see Table 1). Only females that were obviously carrying eggs were chosen for the experiment, because not every female present at a pond is ready to reproduce and

some individuals skip reproduction in a given year (Reyer et al. 2004). Using artificial fertilization (Berger et al. 1994), we crossed each individual with each possible genotype within and between ponds which resulted in 42 different mating combinations (Table 1). For each of these combinations we produced 3 replicates (except for LLR females from pond 011, see above). A total of 120 crossings were carried out and included in the analysis.

On May 21, one day before the artificial crossing, we injected all females with a salmon Luteinizing Hormone-Releasing Hormone (LH-RH; H-7525, Bachem, Bubendorf, Switzerland), which induces ovulation (Mc Creery and Licht 1983, Licht et al. 1987).

The following day (May 22), all males were euthanized with ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma A5040) and subsequently dissected. Their testes were removed and stored in Holtfreter's solution at 4°C until crossings were performed. Shortly before the crossings, one testis was crushed in 15ml filtered pond water with a forceps to produce a sperm solution. Eggs from each individual female were stripped in about equal proportions into several Petri dishes pre-filled with 5ml filtered pond water. Crossings were then performed by adding to each Petri dish 1-1.5ml sperm suspension from the assigned male, which is sufficient to fertilize all eggs. The Petri dishes were subsequently filled with filtered pond water until the freshly fertilized eggs were fully covered to ensure the best possible conditions for embryo development. All crossings were done on the same day. The fertilization success per cross was determined as the proportion of eggs per Petri dish that had rotated their black animal hemisphere to the top (Berger et al. 1994, Reyer et al. 2003).

### *Rearing design*

On May 23 (one day after the crossings), the eggs were transferred from the Petri dishes into 1–liter tubs containing aged tap water and stored in a lab room at approximately 20°C. Unfertilized eggs or embryos that stop development can cause degradation of water quality and were therefore removed during regular checking of the tubs. Water was also changed twice a week for the same reasons.

The larvae were kept indoors until 18 days after fertilization (June 9), when all larvae had at least reached stage 25 (disappearance of external gills according to Gosner 1960). The remaining surviving larvae were weighed, staged and counted. Hatchling survival was determined as the number of hatched larvae relative to the number of fertilized eggs. On the same day, we randomly chose 15 tadpoles from each cross and transferred them to 50l outdoor tubs. At this stage developmental abnormalities, such as curved, bent or shortened tails, asymmetric or inflated bodies and narrow or thickened heads (Ogielska 1994) may appear, but their genetic background and the effect on survival are not yet fully understood. Therefore we also included tadpoles with mild forms of abnormalities for the outdoor raising, i.e., those without any obviously lethal deformations like no swimming or feeding capabilities. Some crosses produced less than 15 viable tadpoles, in these cases we transferred the available amount ranging from just one tadpole to 14 instead. In five crosses however, none of the tadpoles had survived and consequently those crossings were not included into the further analysis. The outdoor tubs had been filled 6 weeks earlier with water, inoculated with phyto- and zooplankton and provided with 1-3 snails (*Lymnaea sp*.) to create a self-sustaining aquatic community (Semlitsch and Reyer 1992, Semlitsch 1993). The tubs were covered with lids, preventing colonization by invertebrate predators. The tubs were arranged in a random design. Tadpoles were fed every other day *ad libitum* with rabbit chow and counted on average every ninth day. Larval survival rates for these crosses were determined as the proportion of tadpoles surviving until August 9.

From August 9 until August 13, we terminated the experiment in Sweden, counted, weighed and staged all surviving individuals and collected blood and tissue from each of the offspring for genotype analysis.

### *Gamete production of genotypes*

All adult frogs and resulting offspring were analyzed with flow cytometry (blood samples) and microsatellite analysis (tissue samples) for final genotype determination as described by Jakob and Arioli (chapter 1 in this publication). By determining genotypes

of all adult individuals and all offspring we could assess which gametes had been produced by the different individuals included in the experiment as parents.

### *Statistical analysis*

If an individual produced more than one gamete type, the frequency was calculated as the number of this gamete type per total number of offspring from this individual. Differences in egg numbers between female genotypes were analyzed with a general linear model using PROC GLM in SAS including snout-vent length as a covariable. Similarly, we tested with a linear regression if fertilization success of males was influenced by male size. The category "crossing type" indicates if the parents that were mated originated from the same pond ("within ponds") or from different ponds ("between ponds"). The early developmental variables (fertilization success and hatchling survival) were analyzed with general linear models using PROC GLM in SAS to test for the effects of crossing type, female genotype, male genotype and their interactions. Genotypes were nested within pond to account for different origin of the same genotypes. Because of missing genotypes, the experiment was not completely balanced and we therefore used Type III sums of squares. Fertilization success and hatchling survival were measured as proportions and thus arcsine-square root transformed prior to analyses (Stahel 1995). Additionally, a t-test was performed to specifically test for the effect of crossing type for a sub-dataset including only LL and RR offspring genotypes. Since indoor rearing density for the different crosses was very variable, we tested with a linear regression for the effect of density on developmental stage and weight of young larvae before they were put into the outdoor tanks.

The effect of offspring genotype, crossing type and their interaction on outdoor larval survival after 12 weeks of development was tested in a general linear model. Similarly, we tested the developmental stage and weight after 12 weeks of development in a general linear model for the effect of offspring genotype, crossing type and their interaction. For this reason, we analyzed cross means, including only the crossings where most of the offspring had the same genotype. Crossings from males with unusual sperm production (see below) were not included in the analysis.

Statistical analyses were carried out with SAS 9.1.3 SP3 for Windows (SAS Institute Inc. 2002-2003). Graphs were produced using SigmaPlot 2002 v8.02 for Windows (SPSS Inc. 1986-2001).

## Results

### *Gamete production, egg numbers & sperm production*

For each parent individual we analyzed between 25 and 56 offspring (pooled from all crosses in which the specific parent individual participated) in order to determine the parents' gamete production and to ensure that rare gamete types were picked up. However, there was one exception (LR male from pond 001), where only two larvae could be analyzed due to extremely low fertilization success of this male (for details see Table 2).

Of the four LLR females, one produced only L eggs, and three other females made mainly L eggs with very few diploid LL eggs (2.6% – 10.7%). All six LLR males from both ponds produced exclusively L sperm.

In total, we investigated six LRR females. Four of them made solely haploid R eggs, one LRR female produced very few diploid RR eggs (4.3%) beside haploid R eggs, and among the gametes of one LRR female we were surprised to detect one LR egg (3.6%) beside the commonly produced haploid R eggs. Since LRR males are very rare in the investigated area, we could only include three individual males in the experiment which all passed on purely R sperm to their offspring.

LR individuals were present in all three investigated ponds and were therefore most numerous in the sample. Of a total of ten LR females, four produced purely diploid eggs, the others produced also haploid R eggs in a frequency ranging from 2.8% - 48.8% (Table 2). Four of the altogether nine LR males showed an unexpected gamete production, as they produced not only the expected haploid R sperm but also haploid L sperm and diploid LR sperm, in some cases simultaneously. This concerned all three LR frogs from pond 001, the fourth male originated from pond 089. Fertilization success of

one of these LR males from pond 001 was especially low, resulting in only two larvae that could be used for genotype analysis. A reliable conclusion about the relative gamete production of this male (proportions of L vs LR sperm) is therefore not possible. All LR males from pond 011 as well as the two remaining diploid males from pond 089 passed on purely R sperm.

From the 4 triploid individuals that were heterozygous for one of the investigated microsatellite loci we found that both genomes that are present in double copy number were inherited about equally by the offspring (data not shown). This finding supports the results from an earlier experiment (Arioli et al., in prep.). Egg numbers were significantly different between female genotypes (F=6.23, p=0.010), increasing from LLR through LR to LRR, even after including snout-vent length (SVL) into the statistical model, for which egg numbers were also significantly increasing with female size (F=7.08, p=0.017). We also investigated if low fertilization success could be attributed to small males that might not yet have been sexually mature, but we could not find such a relationship (regression $R^2$=0.091, p=0.224). The smallest individual had a rather high fertilization success (91.5%) whereas the biggest individual's success was fairly low (19.9%).

### *Fertilization success and hatchling survival of the different genotypes depending on parental pond of origin*

Female genotypes did not significantly differ in either fertilization success or hatchling survival (Table 3, Fig. 2). On average, 66% of all eggs were fertilized and of those fertilized eggs, another 40% developed into hatchlings. Males on the other hand showed clear differences in their reproductive success in these early stages (Table 3, Fig. 2). Diploid LR males from pond 001 had very low fertilization rates (8%), as well as low hatching success (19%). This translated into only very few surviving tadpoles from these crossings. Such extremely low reproductive success was not found for the same genotype from the other ponds: LR males from pond 089 had an average fertilization success of about 58% and the fertilization success for LR males from pond 011 was even very high (96%). Thus, there was high variability within this genotype, depending on the

pond origin. Triploid LRR males had a high fertilization success (91%), whereas for the LLR genotype it depended again on the pond of origin. Here, males from pond 001 had high fertilization success (87%) whereas LLR males from pond 011 were somewhat restricted in their ability to fertilize (61%). The results for hatchling survival depending on male genotype were similar to those for fertilization success (Table 3, Fig. 2).

The interaction between male and female genotype had no significant influence on fertilization success and hatchling survival, indicating that none of the possible combinations between the two different parent genotypes was substantially better or worse than another (Fig. 3).

The overall fertilization success was not significantly differing between crossing types, i.e., crosses performed with parents from the same pond or with parents from different ponds ($t_{fertilization}$=-0.72, p=0.472; $t_{hatching}$=-0.64, p=0.525), as shown in Fig. 4. Since the effect of differing pond origin might be most severe in the homotypic offspring genotypes LL or RR where deleterious mutations in homozygous (same clone) or heterozygous states (different clones) are not countered by the influence of another genome, we also tested for these two genotypes if crossing type is influencing reproductive success in early life stages, but there were no significant differences in fertilization or hatchling survival ($t_{fertilization}$=0.04, p=0.966; $t_{hatchling}$=0.33, p=0.741; Fig. 5a).

The stage and weight of the larvae at this point in development are both negatively density dependant on the number of surviving tadpoles left in the crossing (both $R^2$>0.1854, p<0.001) and therefore only indirectly influenced by the crossing type via survival probability of the offspring.

### *Larval survival, stage and weight at later developmental stages depending on offspring genotype*

Due to the partly unusual gamete production, some offspring exhibited also uncommon genotypes; besides the five expected genotypes (LR, LLR, LRR, LL and RR), we also found thirteen tetraploid LLRR larvae, two LLL larvae and one tadpole each of the genotypes RRR, LLLR and LRRR, respectively.

Not all tubs consisted of offspring with one uniform genotype, which was mostly due to those males that produced more than one sperm type, and due to the LR females which simultaneously produced haploid and diploid eggs. Since larval survival was measured per tub, we restricted the analysis of the effect of offspring genotype on larval survival to tubs that held only one genotype or had only very few offspring of the second genotype. After 12 weeks of development, there was a clear significant difference in survival between genotypes (Table 4, Fig. 5a). Although both "parental genotypes" LL and RR tended to survive worse than the hybrid genotypes (LR, LLR and LRR), pairwise comparisons showed that only RR genotypes were significantly different from the hybrid genotypes (all $p<0.05$). Neither crossing type nor the interaction between crossing type and offspring genotype was significant. Offspring genotypes were also differing significantly in stage and weight at the end of the experiment (Table 4, Figs. 5b and c). Offspring with RR genotype was developing much slower and were also lighter after twelve weeks of development. LL tadpoles tended also to develop slower, but were heavier than the rest. The higher weight was dismissed as artifact, however, because the larvae tend to get lighter as they approach metamorphosis.

In 67 of the 120 crossings we observed at least one tadpole that was lagging clearly behind in development, but in most cases, they were not of a different genotype than the rest of the crossing's offspring (data not shown).

## Discussion

### *Influence of pond origin on the offspring formation*

Results from field investigations in Sweden showed that among the adult population no frogs with parental genotypes (LL or RR) were found (Jakob et al., chapter 2 in this publication), but there were decreasing amounts of homotypic genotypes in early developmental stages. In an earlier artificial crossing experiment with Swedish hybrid frogs we found that there is a tendency of parental genotypes to survive better if parents stem from different ponds (Arioli 2007). Results from the present study showed, however, that the crossing type, i.e., whether the parents originated from the same

pond or from different ponds, did not influence the successful formation or chance of survival of any offspring genotype in our experiment. Fertilization success as well as hatchling survival for offspring stemming from crossings within the same pond were similar to those with parents from differing ponds.

It has been shown for a Swiss LE-population system that the success of matings between hybrids depends on the origin of the parents (Vorburger 2001, Guex et al. 2002). Frogs from different ponds often possess different hemiclones (i.e., they originate from different primary hybridizations) so that the R-genomes of different populations exhibit different deleterious mutations. Hence, when two hybrid frogs from the same pond mate, the offspring dies due to accumulated deleterious mutations occurring in a homozygous state. For the Swedish pure hybrid populations the premises are slightly different. Only recently has it been shown for triploid individuals that recombination between the genomes that are present in double copy number is possible (Arioli 2007), although it is not resolved yet if recombination is rare or rather the rule in the gametogenesis of triploids. Offspring with a parental genotype (LL or RR) usually originate from at least one triploid parent and therefore the inherited genome is not strictly clonal. Consequently, we would not necessarily expect the same outcome as in LE-systems. However, genetic diversity in the area of Southern Sweden was found to be very low compared to other regions (Arioli 2007), which again elevates the risk of two genomes having the same deleterious mutations expressed in a homozygous state, even if recombination can take place. The result that crossing type has no influence neither on the formation nor the success of offspring genotypes suggests that even though there are clear differences in population composition between ponds, these populations do not differ in their genetic attributes. If genotype compositions in pure hybrid populations are driven by ecological influences or even stochastic effects rather than differential "population systems" similar to LE- and RE- population systems in Central Europe, we would expect such an outcome even in a cross-pond experiment (see also Jakob and Arioli, chapter 3 in this publication).

### *Fertilization success and hatchling survival of the different genotypes depending on parental pond of origin*

Although fertilization success and hatchling survival were not influenced by crossing type (there was no significant difference between crosses from within the same pond and the ones between different ponds), we have found large differences between male genotypes in both fertilization success and hatchling survival. Crosses fertilized by LR males had rather poor early survival. However, not all males from this genotype did poorly. It was mostly the three LR males from pond 001 as well as one LR male from pond 089 that accounted for this low fertilization success and hatchling survival. Such a high variability in fertilization success for diploid hybrid individuals is not surprising. It has been shown earlier that some LR males are almost completely sterile whereas other males from the same population show normal fertilization success (Günther 1975, Günther 1990); this was attributed to the hybrid status and the associated complex process of gametogenesis (Tunner and Heppich-Tunner 1991). Ogielska and Bartmanska (1999) also noticed that gonad development and germ cells in *R. esculenta* are often abnormal. But all these abnormalities are externally not visible.

When examining the gamete production of these four unsuccessful males more closely we found that they produced not only the "expected" R-sperm but also L-sperm and diploid LR-sperm. This striking aberration in their gametogenesis seems to be the reason for their low reproductive success. There are obviously geographical areas where the induction mechanism of genome exclusion is not uniform, possibly due to one or several missing genes (Hotz et al. 1985, Mancino et al. 1987). Variation in gametogenesis is, for example, known from RE-systems, where diploid LR males produce both types of haploid gametes (L and R) (Vinogradov et al. 1991). Additionally, the occasional formation of diploid sperm in hybrid males has been described earlier (Uzzell et al. 1977, Rybacki 1994), but has usually been dispatched as unimportant due to the obvious disadvantages in velocity compared to haploid sperm. The high proportion of diploid sperm as well as the simultaneous formation of three different gamete types in LR males in our investigation is quite astonishing. The large amount of

diploid sperm also resulted in many tetraploid offspring when LR males were mated with diploid LR females. In fact, tetraploid adults are known in these populations in Sweden (Jakob et al., chapter 2 in this publication) and although they are not very common, they do seem to survive well and could be important for a further step in speciation of the hybrid taxon (Alves et al. 2001, Vrijenhoek 2006). Considering that tetraploid offspring can only be formed when diploid sperm is produced, these diploid LR males are potentially very important for the evolutionary perspective of the pure hybrid populations in Southern Sweden, although their individual reproductive success is low. Unfortunately, it is not known yet which gametes are produced by tetraploid individuals, but in the aforementioned study we found that both sexes are formed even though the frequency of tetraploid frogs in the total sample was low. Results for the other the other two male genotypes (LLR and LRR) showed not as high a variability in fertilization success and hatchling survival as for the diploid males, although LLR males from pond 011 were slightly worse in fertilization than LLR males from pond 001. For male LRR, no comparison between ponds was possible because males of this genotype are very rare due to a high female sex bias (Jakob et al., chapter 2 in this publication). The mechanism for gamete production in triploid individuals is different than in diploid males (reviewed in Graf and Polls Pelaz 1989) which consequently resulted in different findings. While genome exclusion in diploid males was not always consistent and resulted in three different gamete types, triploid males always produced sperm containing the genome that is present in double copy number (i.e., L sperm from LLR males and R sperm from LRR males). These results confirm findings from an earlier experiment (Arioli 2007) as well as other studies (Vinogradov et al. 1990) and indicate that the cytogenetic mechanism of hemiclonal inheritance is simpler in triploids than in diploids. Besides the inheritance pattern as seen in Swedish triploid males, there are also exceptions to the rule. Tunner (2000) showed in a Hungarian population that triploid LLR males were producing exclusively diploid LL-sperm. Unfortunately, our sample did not include any animals that were heterozygous for more than one microsatellite locus. Therefore, we could not contribute to the findings that there is

occasionally recombination between the homotypic genomes occurring in the germline of triploid water frogs (Arioli 2007).

The female's genotype had no influence on the fertilization success and hatchling survival, indicating that the egg quality from the different female genotypes was similar. There was however a difference in the amount of produced eggs between female genotypes. Body size is naturally influencing the number of eggs carried by a female, but additionally we found that LRR females were carrying proportionally more eggs. This result suggests that LRR females contribute most to the offspring generation if everything else is equal. For Polish water frogs it has been shown that although the number of eggs increases with size in all three taxa *R. lessonae*, *R. esculenta* and *R. ridibunda*, the influence of female size on egg number is not as strong for *R. lessonae* as for the other two taxa (Plötner 2005). Thus it is possible that in pure hybrid populations, LRR females produce relatively more eggs with increasing SVL than the other genotypes, but many more female individuals would be needed to determine the exact relationship between egg number and female size. However, since the crossings were done artificially and as many eggs were stripped from the females as possible, this result has to be treated with care and can not be transferred directly into natural conditions. Reyer et al. (1999) demonstrated that females can adjust the number of eggs laid depending on male partner. Additionally, in an experiment done with Swedish water frogs in 2002 no increased egg number of LRR females was found, though sample size was smaller (Arioli 2007). Produced gamete types of triploid females were similar to the ones in triploid males, with a few exceptions. After analyzing a large amount of offspring we found that many triploid females may rarely produce diploid eggs, revealing that the gametogenesis in these triploid hybrid females is not always straightforward either.

For diploid LR females, it is known from other all-hybrid areas that they can produce haploid and diploid eggs (Berger and Roguski 1978, Günther et al. 1979, Fog et al. 1997). However, the frequency was rarely quantified and explained. In accordance to the 2002 crossing experiment, we found that the proportion of diploid eggs was usually

over 80% (9 of 10 LR females). But in one case (a female from pond 011), the proportion of the two egg types was equal.

Unfortunately, earlier studies describing the formation of two or even three eggs sizes did seldom quantify the amounts of the different types. An exception comes from Berger and Berger (1992) who described for a Polish population the production of 95% haploid eggs containing an R genome and only 5% diploid LR eggs, which is very distinct from what we found in Sweden. There is however no information about the underlying mechanism for the production of two different gamete types and whether it is genetically or environmentally determined. For an individual female, the reproductive success is strongly influenced by the ratio of haploid to diploid eggs as well as by the available male genotypes in the pond. In the Swedish pure hybrid populations it seems more advantageous to produce mainly diploid eggs, since triploid offspring seem to survive just fine, in contrast to diploid RR offspring resulting from the fusion of a haploid R egg with R sperm. Although individual fitness can vary strongly, it has been shown in models that the population can nevertheless be stable for a range of different egg type ratios (Som and Reyer 2006).

*Larval survival and development of offspring genotypes*

A consequence of the uncommon gamete production of several genotypes in these populations is the occurrence of all kinds of unusual genotypes among the offspring in the experiment, such as triploid parental genotypes (LLL and RRR) or different types of tetraploid larvae (LLRR, LLLR and LRRR). It was not surprising to find them in the experimental sample in low numbers, because we have also found occasionally such unusual genotypes in larval samples from natural ponds (Arioli and Jakob, chapter 6 in this publication). In contrast to the field samples, however, these genotypes were still alive at the late larval developmental stage (≥ stage 40) in the experiment. Although they externally looked no different than the other genotypes at this stage, we do not know if they would have survived through metamorphosis, which is very challenging for the organism.

Survival during the first weeks of development was rather high and not differing between the offspring genotypes (data not shown), but it became noticeable towards metamorphosis (± after 12 weeks of development) that offspring with a parental genotype (LL or RR) survived worse than the hybrid genotypes. Although raising conditions were benign, these genotypes were at a survival disadvantage. Similarly, the surviving larvae of these genotypes were developing slower than the ones of other genotypes, which resulted in a longer time until metamorphosis. The timing of metamorphosis can strongly influence survival at later life stages (Altwegg and Reyer 2003). The earlier a tadpole undergoes metamorphosis, the more time there is before hibernation to feed and grow. Since the investigated area in Sweden is located further north than most other *R. esculenta* populations, the first autumn period after metamorphosis is even shorter and might be much more important than elsewhere. Despite the lower survival and slower development of these LL and RR genotypes compared to the hybrid genotypes, they were still present at the late larval stages in the experiment at considerable proportions and not at all missing as assumed from the adult samples in natural habitats. Homotypic genotypes are also formed in natural habitats, but under higher selection pressure, they die off more quickly (Arioli and Jakob, chapter 6 in this publication).

## Conclusion

Results from this experiments demonstrated that the pond of origin of the parents had no direct influence on the successful formation or the chance of survival of any offspring genotype. Nevertheless, particular genotypes from different ponds differ in their gamete production, fertilization success and hatchling survival. Thus, there are no valid general assumptions that could be used to predict the stability and evolutionary perspective of pure hybrid populations, which complicates the elaboration of models. We clearly demonstrated that the absence of LL and RR genotypes among adults in natural pure hybrid populations can neither be attributed to non-formation at the larval stage, nor to genetically induced metabolic failure that prevents reaching

metamorphosis, but has to be caused by natural selection pressures during larval development.

## Acknowledgements

We are grateful to Bo Baldetorp and Maria Johansson of the oncology department of Lund University Hospital for granting us access to their FACSCalibur flow cytometer. We thank Eline Embrechts for help with the catching and crossing of the frogs and Christoph Vorburger for valuable hints concerning hormone treatment. Uli Reyer provided helpful comments on the manuscript. The work was funded by the Swiss National Science Foundation, grant No. 3100-64004.00 to H.-U. Reyer. Catching and experimental crossing permits for Southern Sweden were provided by Länsstyrelsen i Skåne Län (522-18591-02, 522-9286-03, 522-6571-04).

## Author contributions

C.J. and M.A. contributed equally to this work. Both authors carried out all field- and lab work together. MA performed statistical analyses and both authors wrote the paper. Both authors discussed the results and MA commented on the manuscript.

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# Tables

**Table 1:** Mating combinations resulting from the experimental crossing. Population type is indicated in brackets behind pond. We included 3 different individuals per genotype and cross in the experiment with the exception of pond 011, where only one LLR female could be caught and one individual was categorized as a LR instead of LLR after final genotype analysis. A total of 120 crossings were produced. Shaded areas are crosses done within a pond, white areas are crosses done between ponds.

| | | Pond (Type) | 001 (LLR dominated) | | 089 (LRR dominated) | | 011 (genotypes balanced) | |
|---|---|---|---|---|---|---|---|---|
| | | Male genotype (n=) | LLR (3) | LR (3) | LRR (3) | LR (3) | LLR (3) | LR (3) |
| Pond (Type) | Female genotype (n=) | | | | | | | |
| 001 (LLR dominated) | LLR (3) | | 3 | 3 | 3 | 3 | 3 | 3 |
| | LR(3) | | 3 | 3 | 3 | 3 | 3 | 3 |
| 089 (LRR dominated) | LRR (3) | | 3 | 3 | 3 | 3 | 3 | 3 |
| | LR (3) | | 3 | 3 | 3 | 3 | 3 | 3 |
| 011 (genotypes balanced) | LLR (1) | | 1 | 1 | 1 | 1 | 1 | 1 |
| | LR (4) | | 4 | 4 | 4 | 4 | 4 | 4 |
| | LRR (3) | | 3 | 3 | 3 | 3 | 3 | 3 |

**Table 2:** Gamete production of the individual frogs sorted by sex, genotype and pond.

| Sex | Genotype | N = | Pond | L-gametes in % | R-gametes in % | LR-gametes in % | Others in % |
|---|---|---|---|---|---|---|---|
| ♀ | LLR | 34 | 001 | 100.0 | | | |
| ♀ | LLR | 38 | 001 | 97.4 | | | 2.6 (LL) |
| ♀ | LLR | 35 | 001 | 94.3 | | | 5.7 (LL) |
| ♀ | LLR | 28 | 011 | 89.3 | | | 10.7 (LL) |
| ♂ | LLR | 47 | 001 | 100.0 | | | |
| ♂ | LLR | 48 | 001 | 100.0 | | | |
| ♂ | LLR | 43 | 001 | 100.0 | | | |
| ♂ | LLR | 40 | 011 | 100.0 | | | |
| ♂ | LLR | 47 | 011 | 100.0 | | | |
| ♂ | LLR | 41 | 011 | 100.0 | | | |
| ♀ | LRR | 35 | 011 | | 100.0 | | |
| ♀ | LRR | 26 | 011 | | 100.0 | | |
| ♀ | LRR | 46 | 011 | | 95.7 | | 4.3 (RR) |
| ♀ | LRR | 41 | 089 | | 100.0 | | |
| ♀ | LRR | 40 | 089 | | 100.0 | | |
| ♀ | LRR | 28 | 089 | | 96.4 | 3.6 | |
| ♂ | LRR | 45 | 089 | | 100.0 | | |
| ♂ | LRR | 41 | 089 | | 100.0 | | |
| ♂ | LRR | 45 | 089 | | 100.0 | | |
| ♀ | LR | 38 | 001 | | | 100.0 | |
| ♀ | LR | 36 | 001 | | 2.8 | 97.2 | |
| ♀ | LR | 46 | 001 | | 6.5 | 93.5 | |
| ♀ | LR | 39 | 011 | | | 100.0 | |
| ♀ | LR | 36 | 011 | | | 100.0 | |
| ♀ | LR | 32 | 011 | | 18.7 | 81.3 | |
| ♀ | LR | 43 | 011 | | 48.8 | 51.2 | |
| ♀ | LR | 47 | 089 | | | 100.0 | |
| ♀ | LR | 36 | 089 | | 5.6 | 94.4 | |
| ♀ | LR | 34 | 089 | | 5.9 | 94.1 | |
| ♂ | LR | 25 | 001 | 24.0 | 28.0 | 48.0 | |
| ♂ | LR | 2 | 001 | 50.0 | | 50.0 | |
| ♂ | LR | 40 | 001 | 10.0 | 67.5 | 22.5 | |
| ♂ | LR | 38 | 011 | | 100.0 | | |
| ♂ | LR | 52 | 011 | | 100.0 | | |
| ♂ | LR | 45 | 011 | | 100.0 | | |
| ♂ | LR | 47 | 089 | | 100.0 | | |
| ♂ | LR | 38 | 089 | | 100.0 | | |
| ♂ | LR | 56 | 089 | | 89.3 | 10.7 | |

**Table 3:** General linear model for fertilization success and hatchling survival testing the effects of female and male genotype, their interaction and the effect of crossing type (within versus between pond crossings). Significant p values <0.05 are printed in bold letters.

| | | Fertilization success | | Hatchling survival | |
|---|---|---|---|---|---|
| Source of variation | df | F | P | F | P |
| Crossing type | 1 | 0.17 | 0.681 | 0.29 | 0.590 |
| Genotype F (Teich F) | 4 | 0.85 | 0.499 | 0.94 | 0.441 |
| Genotype M (Teich M) | 3 | 68.17 | **<0.001** | 10.37 | **<0.001** |
| Genotype F x Genotype M | 4 | 0.49 | 0.746 | 1.94 | 0.110 |

**Table 4:** General linear model testing for differences in larval survival (per tub), developmental stage (Gosner) and weight of offspring genotypes after 12 weeks of development. Significant p values <0.05 are printed in bold letters.

| | | Survival to week 12 | | Development (Stage) | | Weight | |
|---|---|---|---|---|---|---|---|
| Source of variation | df | F | P | F | P | F | P |
| Genotype | 4 | 4.16 | **0.004** | 7.70 | **<0.001** | 3.52 | **0.001** |
| Crossing type | 1 | 0.06 | 0.811 | 0.00 | 0.996 | 0.15 | 0.703 |
| Genotype* Crossing type | 4 | 0.65 | 0.628 | 1.48 | 0.213 | 1.33 | 0.264 |

## Figures

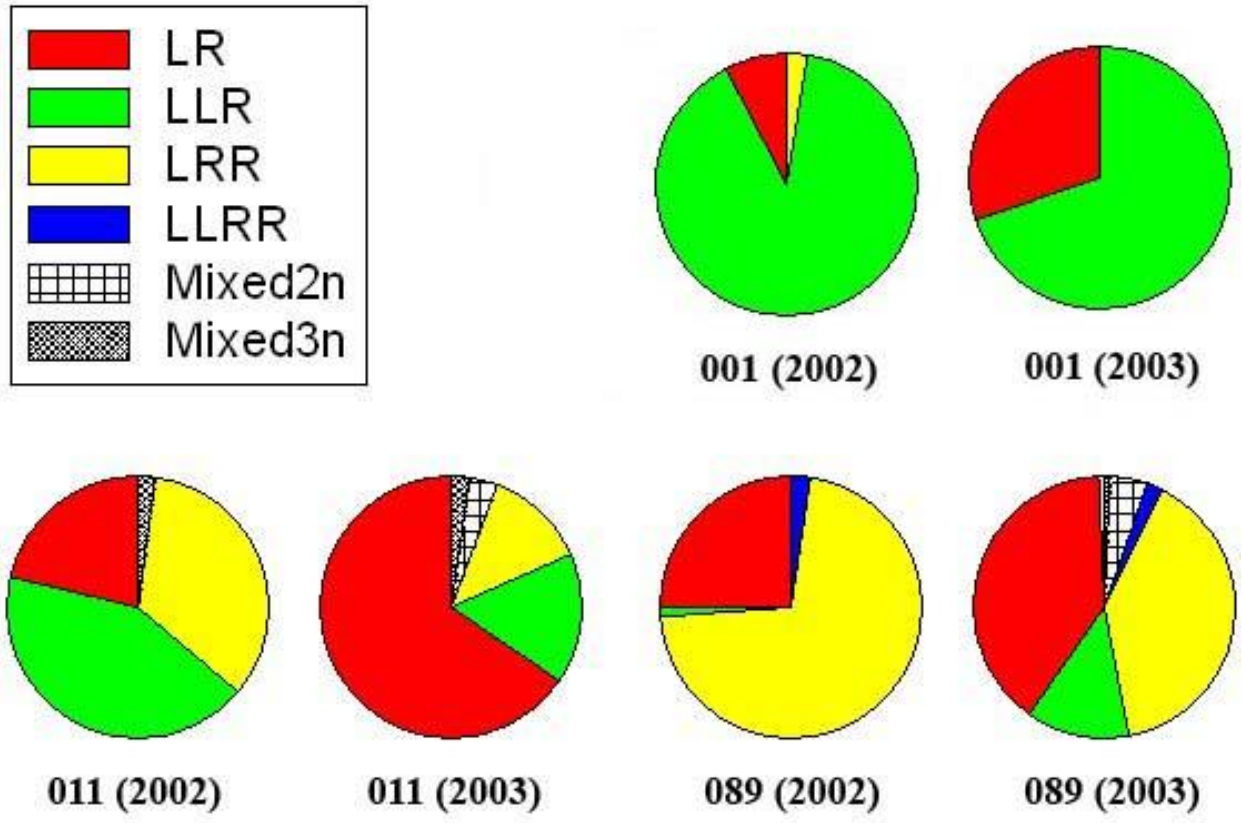


**Fig. 1:** Genotye proportions for the 3 sampled ponds in 2002 and 2003. Mixed 2n/3n represent individuals which showed repeatedly different genotypes in the analysis methods (flow cytometry and microsatellite analysis) indicating an unusual chromosome composition.

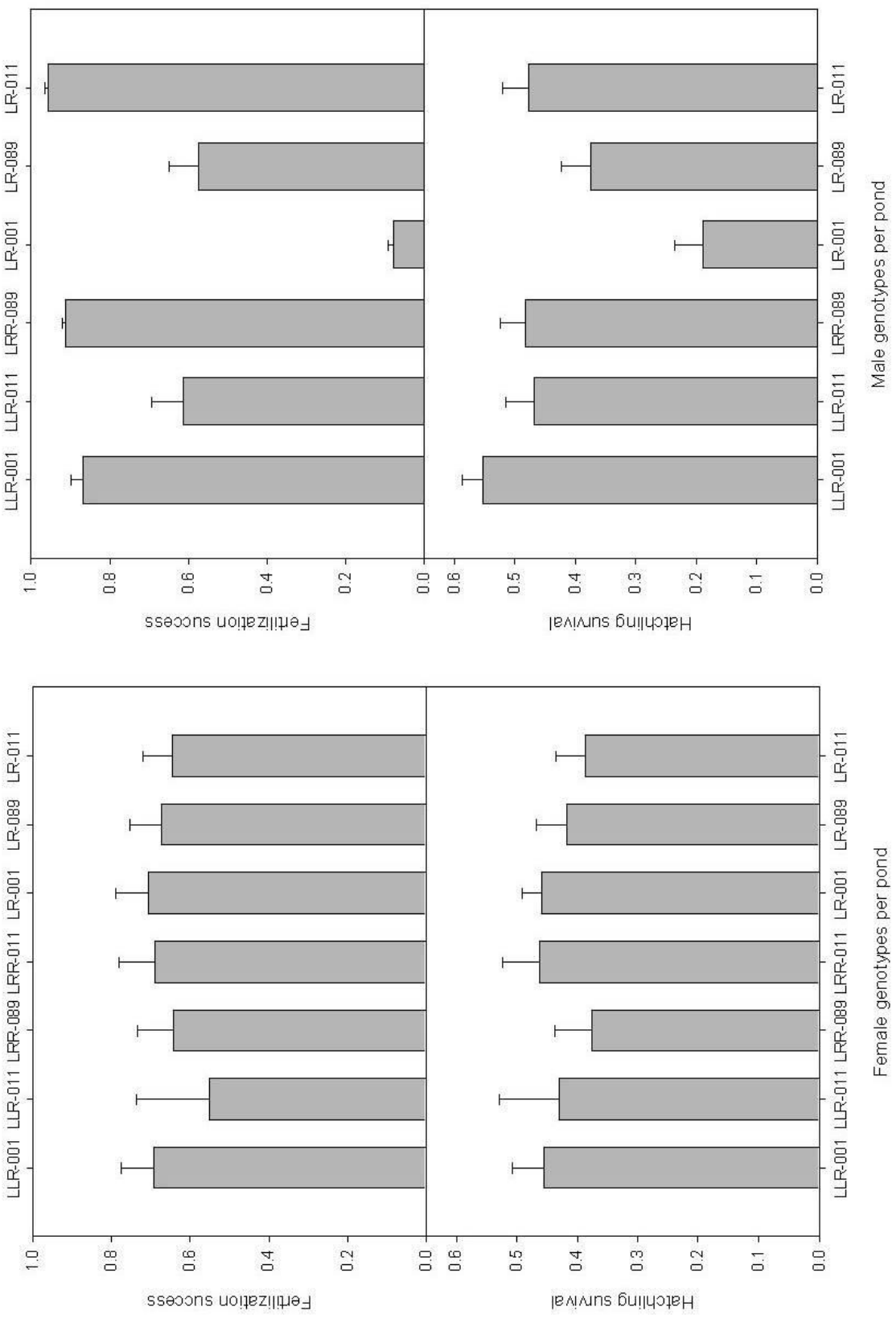


**Fig. 2:** Means in fertilization success and hatchling survival for the different genotypes of females and males listed per pond. Error bars represent ±1 SE.

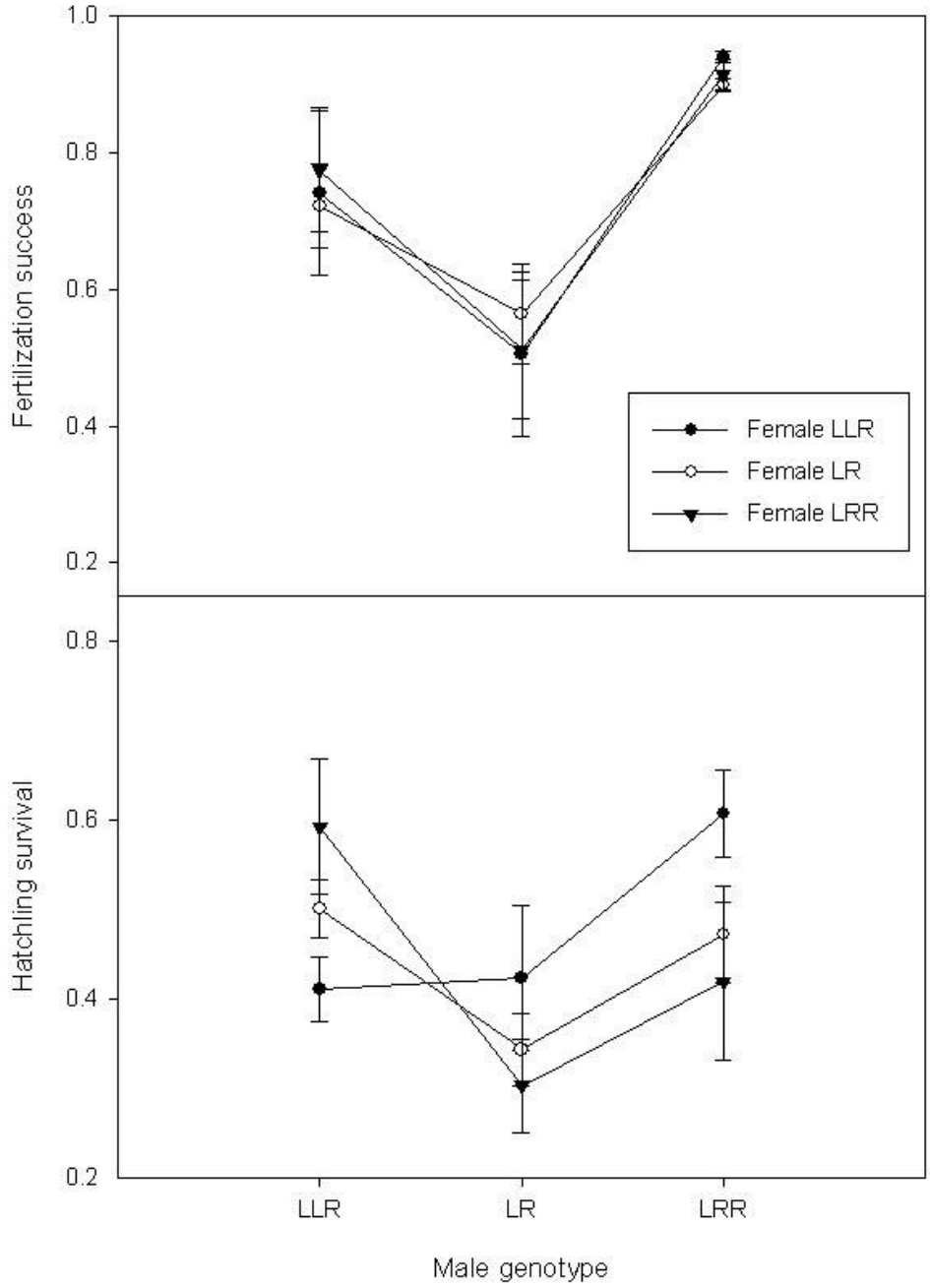


**Fig. 3:** Interaction between male and female genotype for fertilization success and hatchling survival. Values are means and error bars represent ±1 SE.

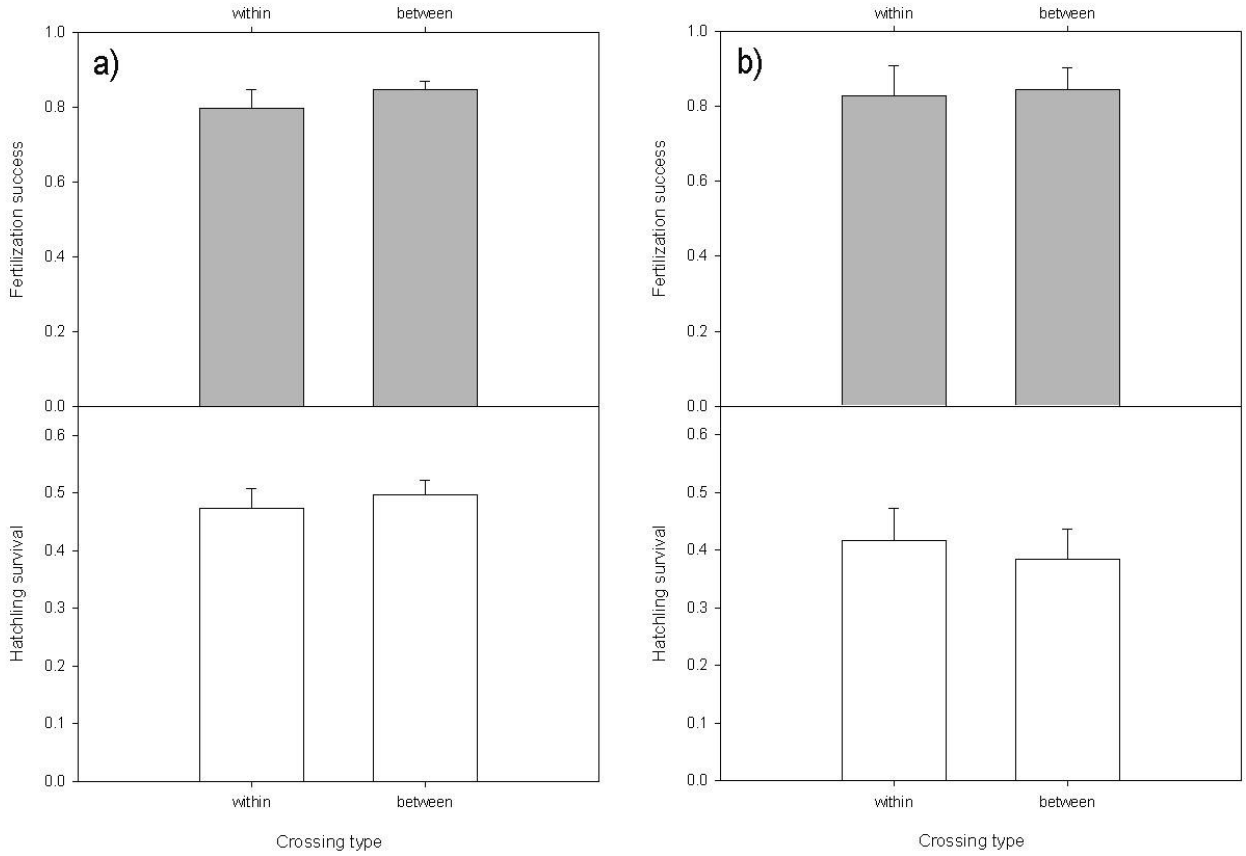


**Fig. 4:** Means (±1SE) of fertilization success (grey) and hatchling survival (white) for crossings with parents from the same pond (within, left bar) and with parents from different ponds (between, right bar); a) represents all offspring types, b) represents only offspring with genotypes LL and RR (parental genotypes)

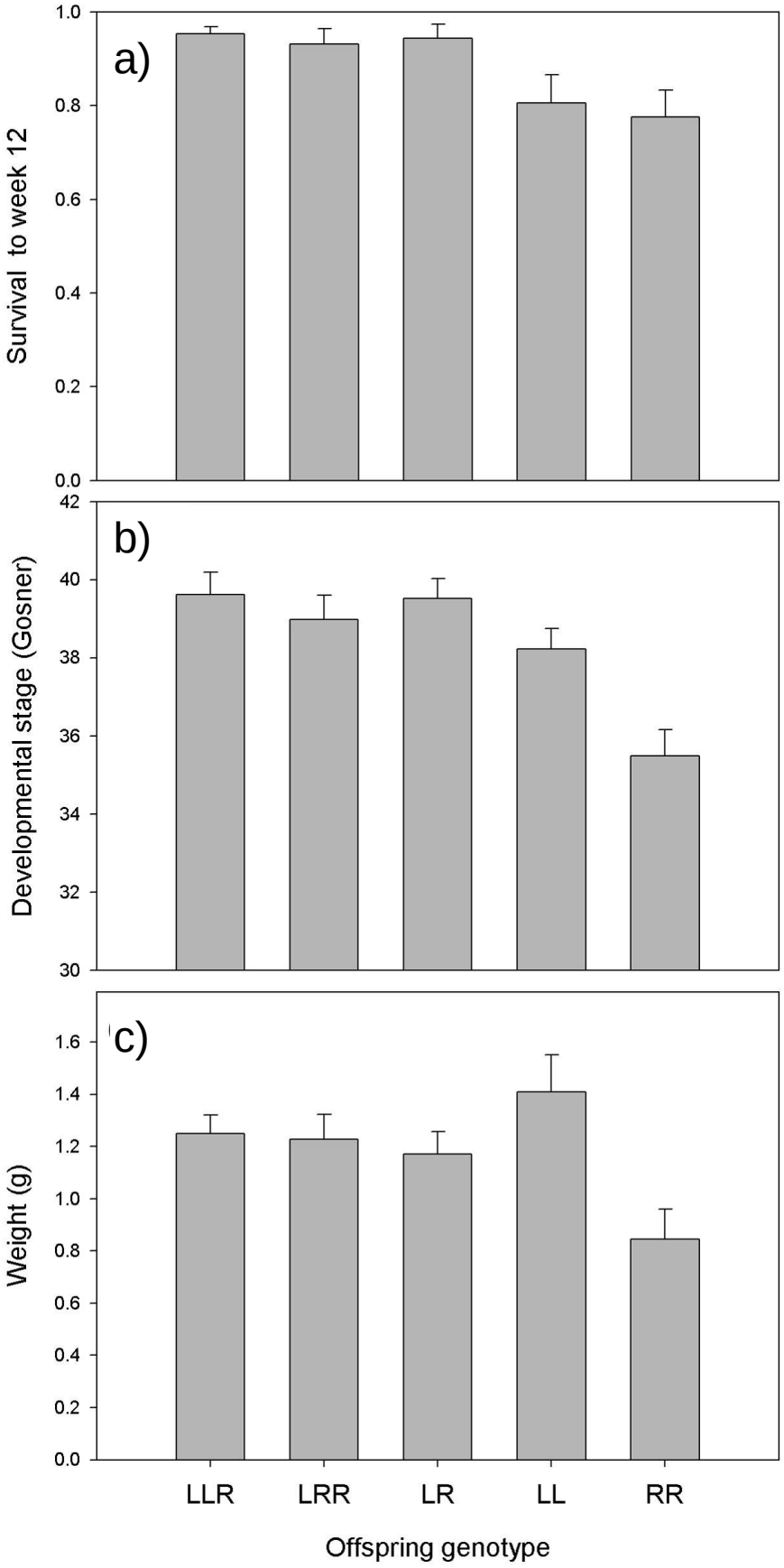


**Fig. 5:** Proportion of tadpoles that survived until week 12 of the experiment in relation to their genotype (a). Differences in developmental stage (b) and weight (c) between the offspring genotypes after three months of development.

# CHAPTER 6

## Genotype composition changes during larval development in pure *Rana esculenta* populations

MARTINA ARIOLI & CHRISTIAN JAKOB

## Abstract

Hybridization between two species leads in most cases to inviable or infertile offspring due to endogenous or exogenous selection pressures. Nevertheless, hybrid taxa are found in several plant and animal genera and some of these hybrid taxa are ecologically and evolutionarily very successful. One example of such a successful hybrid is the water frog, *Rana esculenta* (genotype LR), which originated from the mating between the two species *R. ridibunda* (RR) and *R. lessonae* (LL). At the northern border of the distribution all-hybrid populations have been established, where the hybrid has achieved reproductive independence from its sexual ancestors and forms a self-sustaining evolutionary unit. Based on the gamete production of these hybrids it would be possible that parental genotypes are produced by certain mating combinations, but field sampling has clearly demonstrated that parental forms are absent among the adults.

In order to investigate potential pre- and postzygotic mechanisms that maintain such a pure hybrid system, we sampled several ponds for water frog larvae at different developmental stages. Genotype compositions were then analyzed and life-history differences between the genotypes examined. Half of the individuals in the early egg sample had a hybrid genotype, present also among the adults, the other half were parental genotypes which are not found in the adult population. The frequency of these parental genotypes decreased drastically in the later larval stage, and practically no individuals with parental genotypes were found among the metamorphs. Our finding

supports the hypothesis that mating is random (i.e. no prezygotic selection) and that it is postzygotic natural selection in the ponds that acts against certain genotypes and sustains the adult pure hybrid population.

*Keywords:* all-hybrid populations, post-zygotic selection, hybridogenesis, *Rana esculenta*, larval development, hybrid survival

## Introduction

The western group of Palearctic water frogs is a well studied complex consisting of several different "good" species which can form viable hybrid taxa. The most abundant species in Western Europe are the lake frog (*Rana ridibunda* Pallas, 1771), the pool frog (*Rana lessonae* Camerano, 1882) and the Iberian water frog (*Rana perezi* Seoane, 1885). Additionally, Uzzell and Hotz (1979) described the Italian non-hybrid (*Rana bergeri* Günther, 1985) which occurs only in Italy and resembles *R. lessonae*. There are several other water frog species in Europe, which have been studied, but not as extensively as the ones described above. In addition to the "good" species, three hybrid complexes have been described so far (Plötner 2005). Due to repeated hybridization between *R. ridibunda* (genotype RR) and *R. lessonae* (genotype LL), the edible frog (*Rana esculenta* Linnaeus, 1758, genotype LR) was formed, which is by far the most widespread hybrid taxon and distributed over most of Central Europe (Fig. 1). Another hybrid, *Rana grafi* Crochet et al., 1995, emerges from the mating between *R. ridibunda* and *R. perezi*, but this hybrid taxon occurs only in the region of the Pyrenees. The third hybrid taxon, *R. hispanica* Bonaparte, 1839, originates from the hybridization between *R. ridibunda* and *R. bergeri* and occurs only in Italy.

Possibly due to its large geographical distribution, the complex comprising *R. ridibunda*, *R. lessonae* and their hybrid *R. esculenta* has been extensively studied. The hybrid nature of *R. esculenta* was first shown by Berger (1967, 1970) through biometric analyses and breeding experiments; further investigations revealed that its reproductive mode is hybridogenetic (Tunner 1973). Hybridogenesis involves the premeiotic exclusion of one genome during gametogenesis, the clonal transmission of the other and hence the inheritance of only one parental genome (Schultz 1969). It depends on the geographical region, which genome is transmitted (Berger 1983, Vinogradov et al. 1991) and whether genome exclusion is induced at all or not (Hotz et al. 1985, Guerrini et al. 1997). In Central and Western Europe the L-part of the genome is eliminated and, in order to restore the hybrid condition of the offspring, the hybrid has to mate with the parental species *R. lessonae*. In this area, the hybrid usually co-occurs in mixed populations with

*R. lessonae* (LE-system). For the eastern part of Europe, the reverse pattern has been documented, namely that the R-genome is excluded and the L-genome is clonally transmitted to the offspring. Here, the hybrid normally coexists with *R. ridibunda* (RE-system). Because one genome is always transmitted clonally and the other genome comes from a sexual parent, the reproduction is also called hemiclonal (Dawley 1989). Vorburger (2001) demonstrated that offspring from matings between hybrids usually do not survive due to the accumulation of mutations on the clonally inherited genome, which then occur in the homozygotic form. Therefore, the hybrid is usually forced to coexist and mate with at least one of the parental species. Models have shown that stability in these mixed systems is very sensitive to several factors, such as mating preference, female fecundity and larval performance of the involved taxa (Hellriegel and Reyer 2000, Som et al. 2000, Reyer et al. 2004).

Beside the LE- and RE-systems, all-hybrid populations of *R. esculenta* have been reported in several regions of Europe (Ebendal 1979, Eikhorst 1987, Günther 1991). Most of these pure hybrid populations are located in areas where parental species have also been recorded in populations nearby, and it can not be excluded that occasional parental migrants influence the viability of such pure hybrid populations. But at the northern border of the water frog distribution (Denmark, Sweden) there are isolated areas in which populations are presumed to have no parental forms at all (Ebendal 1979, Fog 1994, Christiansen et al. 2005). This is also true for our study area in Southern Sweden. Here, populations consist not only of diploid hybrids (genotype LR) but also of two triploid forms (LLR and LRR) and very low numbers of tetraploid frogs (Jakob et al., chapter 2 in this publication). Although parental genotypes are supposed to be formed based on the gamete production shown in table 1, extensive sampling has revealed that no parental forms are present among adults (with the exception of one pond where 3 *R. ridibunda* females were discovered).

Little is known about the history of water frogs in this region. Ebendal (1979) reported that green frogs have been described in this area at least since around 1830 (Nilsson 1860) and that they have always been regarded as *R. esculenta* on the basis of

morphometry. But because it is not always trivial to morphologically distinguish between the different water frog taxa (Pagano and Joly 1999), these early observations have to be treated cautiously. Some years later, it was confirmed by albumin electrophoresis that these Swedish water frogs are indeed diploid and triploid hybrids, but it remained unclear which of the two triploid genotypes occurs (Ebendal and Uzzell 1982).

Two scenarios about the origin of these pure *R. esculenta* populations are possible: first, only the hybrid has reached Southern Sweden or, second, parental forms have been present during the initial colonization of this area, but later were outcompeted by the hybrid. Under both scenarios the question how these populations retain pure hybrid status remains unsolved. Because all three hybrid genotypes (LR, LLR and LRR) usually co-occur in the same ponds (Jakob et al. in prep., chapter 2), some matings should result in offspring of the parental genotypes (LL and RR) (Table 1). We tested the following two explanations for their absence among adults:

a) Mating is assortative and only those female x male combinations occur that lead to offspring with genotypes present also among adults.

b) Mating is random and all genotypes are present in the early stages but some are at a disadvantage (ecologically or genetically) and thus disappear during development.

To distinguish between these two possibilities, we sampled twelve ponds for water frog larvae at different developmental stages and analyzed the genotype composition and life-history differences between genotypes.

## Methods

### *Samples and source populations*

In 2003, we sampled twelve different ponds in Southern Sweden (for details see Jakob et al., chapter 2 in this publication) for their genotype composition at three different larval stages: egg stage, tadpole stage and metamorph stage. The aimed target

numbers per pond were: 5-7 egg clutches, 25 tadpoles and 20 metamorphs. However, these sample sizes were not always achieved due to reasons mentioned in the results section; for actual sample size see table 2. Genotypes were determined from blood and tissue samples via flow cytometry and microsatellite analysis (see below). For the egg stage, we collected egg clutches in each pond at the beginning of June (June 1 – June 10) and raised a subsample of 15 individuals per clutch at Stensoffa, the field station of the University of Lund, under *ad libitum* food conditions until July 22. The upbringing of these eggs to tadpoles was necessary because analyzable amounts of blood and tissue can only be collected once the tadpoles have reached a certain size (~ 50 days old). Approximately 6 weeks after the first sampling (July 16 – July 21), samples for the tadpole stage were collected from the same 12 ponds by catching a random sample of tadpoles with a dip net. Both sets of tadpoles (those raised from the eggs and those sampled from ponds) were staged for their development according to Gosner (1960) and then killed with a solution of 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, 5 g/l, Sigma A5040), because it was not possible to obtain enough blood from living animals. Tissue was collected by cutting off part of the tadpole tail. For the metamorph stage, individuals were caught by hand between August 5 and August 12 in each of the ponds. From every metamorph we took a toe clip for microsatellite analysis and a blood sample for flow cytometry analysis. Blood was obtained by cutting the web of a hind foot and collecting the emerging drop with a heparinized capillary. Additionally, we measured snout-vent length and weight of these metamorphs to check for possible differences in development and size between genotypes. All blood samples were stored in a FRC-solution and all tissue samples were kept in 70% ETOH until lab analysis for the genotype determination was done. The following year (2004) during the sampling of the adult population in the twelve ponds we occasionally encountered juveniles (1-year old) and collected tissue as well as blood from these individuals in order to investigate the overall change in genotype proportions through the first hibernation. The number of collected juveniles ranged from 3 (pond 126) to 24 (pond 102) per pond and added up to a total of 149 juvenile frogs.

*Genotype determination*

It was very important for the study to correctly determine the larval genotype; we therefore combined several techniques to obtain an accurate result. We used the flow cytometry protocol described in Jakob and Arioli (chapter 1 in this publication) to determine the ploidy of individuals from their blood samples. Flow cytometry allows distinguishing between LR, LLR, LRR and other ploidy levels, because L- and R-genomes have different amounts of DNA (Vinogradov et al. 1991). Tissue samples were extracted using QIAamp® DNA mini kit (Qiagen). All individuals were screened for variation at seven polymorphic microsatellite loci: Ca1b5, Ca5, Ca18 (Garner et al. 2000), Res16 (Zeisset et al. 2000), Ca1b6, Re1CAGA10, Ga1a19 (Arioli 2007). Two loci (Ca5, Ca18) showed only alleles for the L-genome. The other 5 loci were not species-specific, meaning that they showed alleles for both the L- and R-genome; but at all these loci the different alleles could unambiguously be assigned to either the L- or the R-genome. The microsatellite loci Ca1b5, Ca1b6, Ga1a19 and Res16 showed gene dosage (Christiansen 2005) which - in addition to flow cytometry - provided further information about the exact genotype. If flow cytometry and all microsatellite loci showed the same result, the individual was clearly assigned to one genotype. In some cases, however, the results were unusual or contradictory, even after reanalysis; e.g. flow cytometry indicated triploidy and one or several microsatellite loci showed LLR and the rest LRR. We also found cases where flow cytometry and most microsatellite loci indicated an LLR genotype, but one locus showed only LR, so there was one allele missing. All these cases showing repeatedly contradictory results were categorized as mixed genomic individuals, hereafter called mixed. Such individuals are also, but rarely, found among adults (Jakob et al., chapter 2 in this publication). Therefore we assume that they are not aneuploid animals with additional or missing chromosome fractions, because these would not survive that long. More likely do they have the same number of chromosomes (2n = 26 or 3n = 39) but not the usual composition of L or R chromosomes, e.g., LR: L = 12 and R = 14, instead of 13 each (Ogielska et al. 2004). Such a pattern can arise if irregularities and deviations from hybridogenetic rules occur during oogenesis, which was already suggested by Uzzell et al. (1975). Additionally we

were able to determine genotypes which were triploid but not hybrids (LLL and RRR) and tetraploid individuals (LLRR). Because the triploid parental types were rare (0.4% of the whole sample), we included them for the analysis in the "normal" parental genotypes (LL/RR).

*Data analysis*

For each of the three offspring stages (eggs, tadpoles and metamorphs) and each pond we calculated the proportions of each genotype in the sample, which were then arcsine-square root transformed before analyses. With a general linear model (PROC GLM (SAS Institute 2002-2003)) we then tested the effects of larval stage (eggs, tadpoles and metamorphs) and pond as a random factor on the proportion of the different genotypes. In a two-sampled t-test we analyzed if the proportions of LR, LLR, LRR animals differ before (metamorph sample) and after (juvenile sample) hibernation.

To examine if genotypes have different developmental rates (measured as Gosner stage, Gosner 1960), we tested with general linear models (PROC GLM (SAS Institute 2002-2003)) the effects of genotype and pond on the development of the tadpoles. The analyses were done separately for the tadpoles raised from the egg stage and for the tadpoles caught later in the ponds because their development measurements are not directly comparable. This discrepancy was due to the fact that the two groups were not sacrificed at the same time, and raising conditions for the tadpoles of the egg stage were probably more benign at the field station than for the tadpoles living in the ponds. At the metamorph stage we used snout-vent length (SVL) and weight to examine morphological differences between the genotypes and ponds and tested them in general linear models. For all GLMs we applied post-hoc pairwise-tests (Scheffé's multiple comparison procedure) to investigate which of the genotypes differed.

In order to investigate if certain genetic combinations (haplotypes) are particularly susceptible to mortality during development, translating into a change of haplotype proportions or decrease in number of haplotypes, we analyzed the genetic variance throughout development in an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) as implemented in ARLEQUIN (Schneider et al. 2000).

## Results

### *Sampling*

For several reasons the aimed sample sizes were not achieved in all ponds (Table 2). In pond 032A we found neither egg clutches nor tadpoles or metamorphs, although adult frogs were numerous. The most probable explanation for the lack of offspring lies in the oxygen content of this pond, which dropped dramatically during the season to almost zero and might not have been sufficient for eggs and tadpoles to survive. The clutches that we collected in pond 138 developed poorly, resulting in only 6 surviving tadpoles. For the later stages the aimed target was successfully achieved in this pond. We found no tadpoles in pond 108, probably due to the fact that the pond is very muddy and covered with duckweed (*Lemnaceae sp*.). Tadpoles simply might have been difficult to discover, although present, because later in the season we managed to sample metamorphs in this pond. Despite enormous sampling effort in pond 102, tadpoles were rather scarce and we detected only 4 individuals. The pond is not overgrown like 108, so detection probability is high; but pond 102 has a high abundance of fish, and hence low tadpole survival. In accordance with the rare occurrence of tadpoles we later detected only 1 metamorph in this pond. At the metamorph stage, we were surprised not to find froglets in pond 134, although we visited the pond several times (Table 2).

### *Genotype composition between stages (eggs, tadpoles and metamorphs)*

Pooled over all ponds, the proportions of LLR and LRR hybrids did not differ between the three stages, but the LR hybrids increased significantly from the egg stage to the subsequent stages (Table 3, Fig. 2). The proportion of parental genotypes (LL and RR) significantly decreased from the egg stage throughout larval development until the metamorph stage (Table 3, Figs. 2 and 3). At the egg stage, 25% of the offspring had a RR genotype and 15% had an LL genotype. The proportion was already significantly lower during larval development (RR: 4.8%, LL: 3.3%) and only one RR and no LL individual was found in the metamorph sample. The proportion of mixed individuals was low (6.0%) early in the development, increasing slightly at the tadpole stage (11.0%)

and decreasing again in the metamorph stage (2.3%), although this change was not quite significant (Table 3, Fig. 3). Tetraploid individuals were very rare (2.8%) already at the egg stage, and none were found among the metamorph samples. The genotype analysis of the juveniles of the following year (2004) showed a similar composition as the metamorph sample of the previous year, with the exception of two discovered LL individuals (Fig. 2). When comparing the proportions of LR, LLR and LRR separately between the metamorph and juvenile sample we did the not find any significant differences (t-Test, df = 20, all $t \leq 1.56$, all $P \geq 0.134$).

*Genotype composition between ponds*

Pooled over all ponds we found no significant effect of pond on the proportion of each genotype except for the genotype LR (Table 3). This difference was mainly due to pond 102 which had no LR individual in any of the three samples, but sample size in this pond was anyway very low due to reasons mentioned above.

*Differences in larval development between genotypes*

The individuals that were sampled at the early stages (eggs and tadpoles) were not sacrificed at the exact same time, and the growing conditions were probably more benign for the larvae raised from the egg sample at the field station than for those in the natural ponds. Therefore, it is not meaningful to pool the developmental stage data between these two data sets.

The first sample of tadpoles that were raised from collected eggs showed overall significant differences in development (according to Gosner 1960) between the genotypes (Table 4a, Fig. 4). Pairwise comparisons revealed that offspring with the parental genotype RR (mean stage 33) did not differ significantly from LL and mixed animals (mean stages 35 and 36, respectively; both $P \geq 0.068$), but they were significantly less developed than all four hybrid genotypes (all mean stage ≥ 37, all $P \leq 0.035$). Also, LL offspring developed significantly slower than most other genotypes (all $P \leq 0.022$), except when compared to RR or mixed animals (both $P \geq 0.473$) (Fig. 4). The genotypes LR, LLR, LRR and LLRR did not differ in developmental stage for tadpoles raised from the early egg sample (all pairwise $P \geq 0.85$).

When analyzing differences in developmental stage for tadpoles randomly caught at the ponds later (tadpole stage), the overall difference between genotypes was not significant (Table 4b, Fig. 5). For tadpoles of both data sets (raised from eggs and caught from ponds) there were significant differences in larval development between the ponds (Table 4a,b).

Later in the development, at the metamorph stage, there were only 4 genotypes present, namely LR, LLR, LRR and mixed animals. We found overall significant differences between genotypes regarding snout-vent length and weight (Table 4c, Fig. 6): individuals of the LR genotype were significantly smaller and lighter than the other genotypes (all $P \leq 0.020$) which did not differ in pairwise comparisons (all $P \geq 0.121$). Mixed individuals did not seem to be at a disadvantage in regard to size and weight compared to the other genotypes; on the contrary, they tended to be the heaviest and biggest individuals (Fig. 6). Again, ponds differed in regard to size and weight of their metamorphs (Table 4c).

*Differences in haplotype frequencies*

We did not detect any significant changes in haplotype frequencies between the stages for either the L- or the R- genome. For both genomes, the genetic variance in haplotypes in the sample was best explained by differences among and within ponds (Table 5).

In the L-genome we found eight different haplotypes in total, with one haplotype dominating at all three stages (70%) (Fig.7a). At the egg stage we found seven haplotypes; the tadpole sample showed all eight haplotypes, and in the metamorph sample six haplotypes were still present. Allele diversity in the R-genome was much higher than in the L-genome and resulted in a total of 27 haplotypes, but one haplotype was dominating in all three stages as well (Fig. 7b). The following numbers of haplotypes were found at the three stages; eggs: 19 haplotypes, tadpoles: 22 haplotypes and metamorphs: 19 haplotypes.

## Discussion

Our results show that, in nature, all possible offspring genotypes are produced initially. Therefore, we conclude that there is no or very inefficient assortative mating acting in these ponds.

Among the first sample taken at the egg stage, half of the sample consisted of genotypes that were also found among adults (LR, LLR and LRR), but the other half was composed of unusual genotypes, i.e. those occurring among adults only rarely (LLRR and mixed individuals) or not at all, such as diploid and triploid parental genotypes (LL, LLL, RR and RRR). The existence of LL and RR offspring suggests that the frogs do not choose their mating partners in order to avoid producing offspring with inviable genotypes, while the existence of LLL, RRR, LLRR and mixed individuals indicates that the occasional formation of unusual gametes such as diploid sperm complicates a potential mate choice system.

Assortative mating has been studied in the water frog complex before, mainly in the LE-system. In this system, it is advantageous for both taxa (LL and LR) to mate with a *R. lessonae* individual to optimize reproductive success. Abt and Reyer (1993), Roesli and Reyer (2000) and Engeler and Reyer (2001) experimentally showed that, when given a choice between LL and LR males or their calls, LR and LL females both preferred LL males. Males on the other hand did not discriminate between female genotypes, which reflects the lower male than female investment into reproduction and, hence, lower fitness costs arising from wrong matings. It has recently been shown that the lack of male choosiness can be easily explained theoretically by several factors such as overlap in size distribution of the females or relative abundance of both female taxa (Schmeller et al. 2005). In the pure hybrid populations all three occurring genotypes are very similar and definitely overlapping in their morphological appearance, especially in size (Jakob and Arioli, chapter 1 in this publication). This is not surprising, considering that they all are hybrids and therefore intermediate between the parental species in their morphological features, male vocalization and other traits. Hence, it might be difficult for a frog to choose a certain hybrid genotype over another based on morphology or

vocalization. To test whether discrimination is possible, choice studies for the pure hybrid systems are definitely needed, and they are presently underway (Rondinelli 2006).

The occurrence of LL and RR genotypes among the offspring can plausibly be explained by certain mating combinations alone (Table 1). However, the existence of triploid parental (LLL and RRR) and tetraploid offspring genotypes, let alone mixed individuals, is somewhat more difficult to explain. It has been shown earlier that diploid sperm can be produced in some water frog populations (Uzzell et al. 1977, Rybacki 1994, Tunner 2000), but it is assumed to be disadvantageous in terms of reproductive ability compared to haploid sperm. Jakob and Ariloi (chapter 5 in this publication) found in a crossing experiment with Swedish hybrid frogs that the diploid males produced, among haploid L and R sperm, also diploid LR sperm. Although this seems to be the exception, it shows that hybrids can produce other gametes than the expected ones and, thus, enhance the uncertainty of the outcome when choosing a partner based on its genotype. So even if these hybrids had evolved the ability to discriminate genotypes, the outcome of mate choice in terms of the resulting offspring would hardly be predictable. Moreover, diploid females can produce haploid and diploid eggs at the same time (Berger 1979), which leads to two very different outcomes when mated to individuals producing R sperm (RR or LRR).

In addition to potential proximate constraints on effective mate choice, there are ultimate reasons why in pure hybrid populations mate preferences are unlikely to evolve, even though Table 1 seems to suggest that individuals would benefit from avoiding matings that lead to inviable offspring, such as LLR x LLR matings resulting in LL. In a theoretical model for LLR/LR populations, Som and Reyer (2006) have recently tracked the evolutionary fate of a potential mate preference mutation. A preference for diploid LR males on the successfully propagated L gamete of a triploid LLR female would result in LLR x LR matings and produce diploid LR daughters with a preference for LR males. This is the wrong ploidy preference for all cases where LR female produce haploid eggs because it results in inviable RR offspring (Table 1). Successfully

reproducing diploid LR females on the other hand produce both, diploid LR daughters that should choose LLR males and triploid LLR daughters that should choose LR males. A preference for a certain male ploidy would, thus, always be detrimental to the inclusive fitness of one of the daughter strands.

In conclusion, assortative mating in pure hybrid frog populations does not exist or work. Consequently, many different offspring genotypes are produced in natural ponds, and the absence of LL and RR among adults must be due to postzygotic selection, i.e. differential larval survival.

Among the tadpoles that were raised from collected eggs, the parental genotypes LL and RR developed significantly slower than the others. Because the raising conditions were the same for all genotypes this could hint at genetic problems which arise because two clonal genotypes with possible lethal mutations are paired and these mutations are expressed at some point during larval development. Similarly, we found in a crossing experiment done with *R. esculenta* from the same Swedish pure hybrid populations that under benign experimental conditions, LL and RR tadpoles developed slower than the other genotypes which translated into a longer time until metamorphosis and lower weight at metamorphosis. Nevertheless these parental genotypes survived in the lab at least until after metamorphosis (Arioli 2007). Likewise, survival in the lab until froglet stage of LL and RR offspring from a pure hybrid population was also observed by Berger (1988), but these genotypes (LL and RR) were not present in the pond at a later developmental stage. This is in contrast to results from a crossing experiment with diploid *R. esculenta* individuals from mixed LE-populations, where Vorburger (2001) showed that the resulting RR offspring from parents of the same hemiclone survived until at most 35 days after fertilization and that they usually showed severe morphological deformations. This experiment was done entirely in the lab which excludes most of the environmental selection on these genotypes and indicates that these tadpoles died due to lethal mutations which are at a homozygous state.

At later stages of the development (i.e., after tadpoles had already been exposed to the natural selection regimes of the different ponds) we found that the unusual genotypes

that represented half of the sample early at the egg stage were much less abundant (only about 20%). From the decline in percentage of parental genotypes during development it is obvious that these genotypes must have a higher mortality than the others. Parental genotypes were found in all eleven ponds in the first sample, whereas among the tadpoles only five ponds still had parental genotypes. Since ponds are ecologically very different it is therefore difficult to identify specific environmental factors that cause this reduction. Under the standardized raising conditions at the field station, larvae with parental genotypes had a slower developmental rate compared to the other genotypes. In nature, such a developmental disadvantage could lead to increased mortality due to stronger competition among genotypes and/or higher predation pressure on smaller than on larger tadpoles. However, those tadpoles with the LL and RR genotypes that had survived to the tadpole stage in nature were not particularly slowed down in their development. The finding that later in development unusual genotypes were less abundant in nature compared to experimental conditions (Arioli 2007) clearly demonstrates that experiments alone do not give a satisfactory picture of what is actually happening in nature. At the metamorph stage there were basically only genotypes left that are also found among the adult frogs. Surprisingly, we also still found relatively many mixed individuals, which exhibited aberrant compositions of L and R chromosome numbers. These individuals seem to have no apparent disadvantage compared to the other genotypes, at least not in morphological traits. If they were true aneuploids (i.e. having missing or additional chromosomes) as suggested in other studies (Christiansen et al. 2005), we would not expect such individuals to survive so well to adulthood. There is a great similarity in genotype composition between the metamorph sample from 2003 and the juveniles from 2004, which indicates that the proportion of genotypes stayed stable over the first hibernation period.

The occurrence of unusual genotypes among the eggs in all eleven ponds confirms that the production of those offspring genotypes is not limited to just single ponds but a common phenomenon in this area. It further suggests that both genetic incompatibilities and environment are important during the selection against the

parental genotypes (LL and RR). However, samples sizes per pond are probably too small to draw conclusions about whether and how different pond conditions affect genotype shifts within a population. The result that tadpoles and metamorphs from different ponds differed in development, size and weight indicates that these ponds are variable in their environment and that these differences influence the growth of individuals independent of their genotype.

Ours is not the first study to investigate larval genotypes in all-hybrid populations; but the difficulty with the few earlier findings is that genotypes were usually identified by morphometric measures alone. Such determination, and hence the interpretation of results, is difficult and unreliable, especially in tadpoles or small froglets and in hybrid adults where morphological measurements are often overlapping between genotypes (Jakob and Arioli, chapter 1 in this publication). Morphometric measures have their limits (Pagano and Joly 1999) and even erythrocyte sizes does not always allow clear classification (Schmeller et al. 2001). Therefore, earlier studies could often not reliably distinguish between the different genotypes, especially when analyzing individuals early in the development (Eikhorst 1988). The development of water frog microsatellite markers has enabled scientists to achieve a higher resolution for genetical questions in this system (Hotz et al. 2001, Zeisset and Beebee 2003, Christiansen et al. 2005). Combined with flow cytometry, microsatellite markers enabled us to not only distinguish between the different normal genotypes, but also to detect uncommon types such as LLL or RRR, tetraploid and mixed individuals which are quite common among the early stages. With the help of microsatellite loci we were also able to examine if certain haplotypes are disappearing during development, but this was not the case. Neither did the diversity of haplotypes change during development.

## Conclusion

Our study is the first to investigate the occurrence and differential survival of non-hybrid genotypes among the offspring in truly pure hybrid populations with highly reliable and exact methods. Among the eggs collected in natural ponds we found that

only about half of the offspring had the genotypes commonly found among the adults (LR, LLR or LRR), the other half showed "unusual" genotypes (LL, RR, LLL, RRR, LLRR or mixed). By following the larvae through their development, we revealed that the unusual genotypes disappeared bit by bit and that at the froglet stage, the parental genotypes had disappeared. Our finding supports the hypothesis that mating is random and that selection in the pond acts against certain genotypes, so that the adult population consists of only LR, LLR and LRR genotypes. It is not yet known which factors do impose this selection; to address this question would be very interesting for further investigations. The production of the above mentioned "unusual" genotypes seems to be a huge waste of reproductive potential. However, on a proximate level, similarities between LR, LLR and LRR and somewhat unpredictable gamete production may simply not allow the frogs to distinguish between "suitable" and "unsuitable" mating partners. On an ultimate level, selection for a preference is unlikely to evolve because, due to the genome pathways in this system, suitability constantly changes from diploid to triploid partner and back.

## Acknowledgements

We wish to thank S. Röthlisberger for help with the lab work. The group of R. Zinkernagel at University Hospital Zürich provided us access to their FACSCalibur. B. Baldetorp and M. Johansson of the Lund University Hospital as well as E. Niederer of IBT at the ETH Zürich provided advice and help for flow cytometry analysis. H.-U. Reyer and G. Pasinelli provided helpful comments on earlier versions of the manuscript. This work was supported by Swiss NF grant No. 31-64004.00 to H.-U. Reyer. Catching permits for Sweden were provided by Länsstyrelsen i Skåne Län (522-9286-03).

## Author contributions

M.A. and C.J. contributed equally to this work. Both authors carried out all field- and lab work together. M.A. performed statistical analyses and wrote the paper. Both authors discussed the results and C.J. commented on the manuscript.

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## Tables

**Table 1:** Gamete production in females and males of the three hybrid genotypes and offspring types arising from the nine potential mating combinations in an all-hybrid population of *R. esculenta*. Female LR can produce both diploid eggs and haploid eggs. Genotypes in grey boxes do not occur among the adults in the population although they are initially produced (Jakob and Arioli, chapter 5 in this publication).

| Females \ Males | LR (R) | LLR (L) | LRR (R) |
|---|---|---|---|
| LR (LR) (R) | LRR / RR | LLR / LR | LRR / RR |
| LLR (L) | LR | LL | LR |
| LRR (R) | RR | LR | RR |

**Table 2:** Sample sizes for three developmental stages collected from 12 ponds.

| | Egg stage | Tadpole stage | Metamorph stage |
|---|---|---|---|
| Pond | No. of eggs (clutches) | No. of tadpoles | No. of metamorphs |
| 001 | 51 (5) | 25 | 20 |
| 011 | 51 (5) | 25 | 25 |
| 014 | 52 (5) | 25 | 25 |
| 032 | 61 (7) | 24 | 15 |
| 032A | 0 | 0 | 0 |
| 089 | 56 (6) | 25 | 25 |
| 102 | 53 (5) | 4 | 1 |
| 108 | 53 (5) | 0 | 17 |
| 111 | 56 (5) | 25 | 25 |
| 126 | 58 (6) | 25 | 22 |
| 134 | 45 (5) | 25 | 0 |
| 138 | 6 (4) | 24 | 21 |
| Total | 542 (58) | 227 | 196 |

**Table 3:** Results from a general linear model relating genotype proportions to differences between three stages (eggs, tadpoles and metamorphs) and 11 ponds (pond 032A was excluded from the analysis because no eggs, tadpoles or metamorphs could be detected).

| | Stage | | Pond | |
|---|---|---|---|---|
| Genotype | *F* | *P* | *F* | *P* |
| LR | 7.15 | **0.005** | 3.95 | **0.006** |
| LLR | 2.62 | 0.100 | 1.30 | 0.299 |
| LRR | 0.31 | 0.734 | 1.92 | 0.109 |
| LLRR | 5.22 | **0.016** | 1.81 | 0.132 |
| Mixed | 3.32 | 0.059 | 1.14 | 0.389 |
| LL | 5.50 | **0.014** | 2.00 | 0.096 |
| RR | 7.59 | **0.004** | 1.22 | 0.341 |

**Table 4:** General linear models testing the difference in larval development (Gosner 1960) between the genotypes and the ponds at the two early stages (eggs and tadpoles) and for differences in SVL and weight at the metamorph stage.

| | | | | Developmental stage (Gosner) | | Snout-vent length (SVL) | | Weight | |
|---|---|---|---|---|---|---|---|---|---|
| Stage | Source | N | df | *F* | *P* | *F* | *P* | *F* | *P* |
| a) Eggs | Genotype | 306 | 6 | 15.52 | **<0.001** | - | - | - | - |
| | Pond | 306 | 9 | 4.31 | **<0.001** | - | - | - | - |
| b) Tadpoles | Genotype | 144 | 6 | 1.48 | 0.189 | - | - | - | - |
| | Pond | 144 | 9 | 18.47 | **<0.001** | - | - | - | - |
| c) Metamorph | Genotype | 194 | 3 | - | - | 5.80 | **<0.001** | 3.75 | **0.012** |
| | Pond | 194 | 9 | - | - | 49.28 | **<0.001** | 23.37 | **<0.001** |

**Table 5:** Variance component from an analysis of molecular variance (AMOVA) for the two genomes (L and R) in relation to the three stages (eggs, tadpoles and metamorphs) and pond within stage.

| Genome | Source of variation | df | Sum of squares | % variation | *P* |
|---|---|---|---|---|---|
| L | Among stages | 2 | 0.581 | -0.82 | 0.780 |
| L | Among ponds within stage | 28 | 24.334 | 7.99 | **< 0.001** |
| L | Within ponds | 1068 | 232.459 | 92.82 | **< 0.001** |
| R | Among stages | 2 | 2.722 | -1.90 | 0.890 |
| R | Among ponds within stage | 27 | 107.853 | 21.60 | **< 0.001** |
| R | Within ponds | 1037 | 399.084 | 80.30 | **< 0.001** |

## Figures

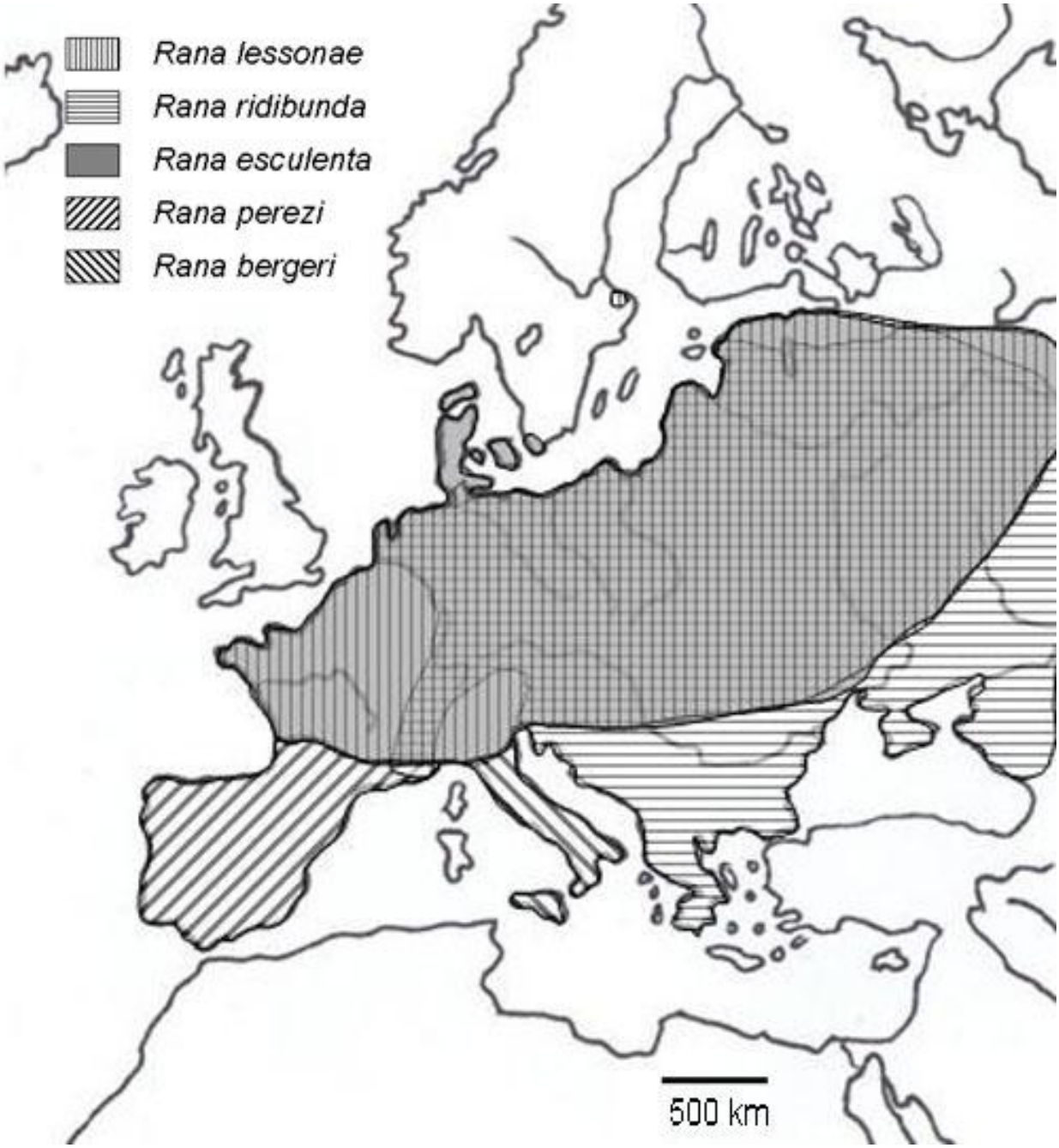


**Fig. 1:** Map of the distribution of the Western European water frogs: *Rana lessonae*, *Rana ridibunda*, *Rana esculenta*, *Rana perezi* and *Rana bergeri*, reviewed in Günther (1990) and Graf and Polls Pelaz (1989).

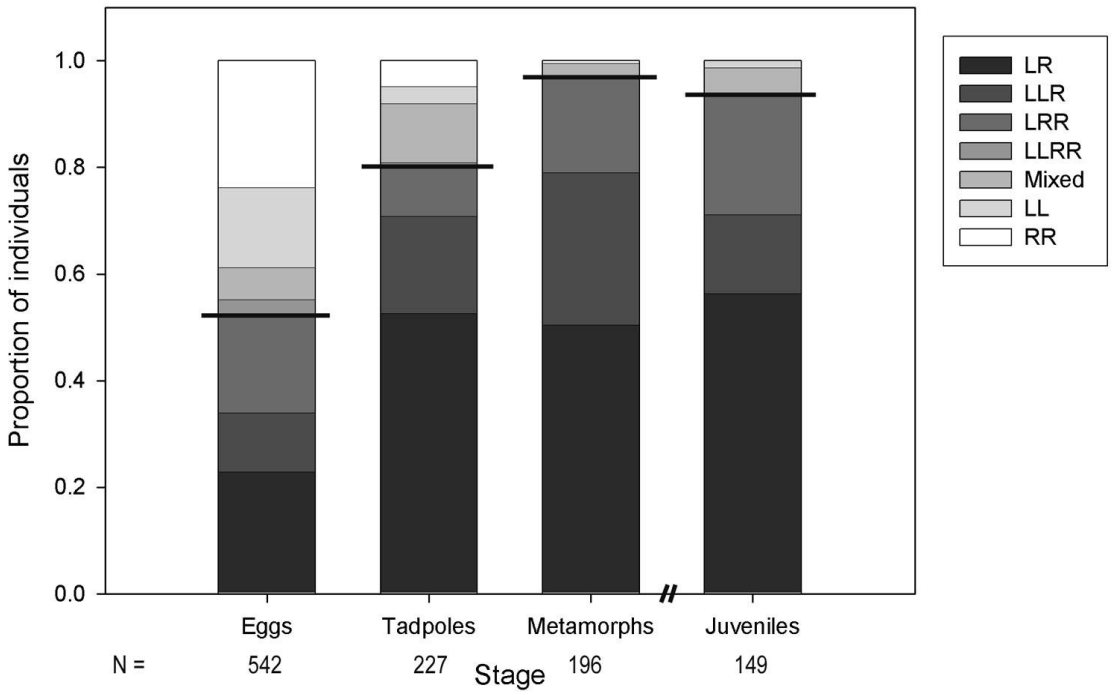


**Fig. 2:** Proportions of different genotypes at the three stages in 2003 (eggs, tadpoles, metamorphs) and for the juveniles caught in 2004. The solid line separates the genotypes which occur commonly among adults (LR, LLR, LRR; below line) from the unusual genotypes (LLRR, mixed, LL and RR; above line).

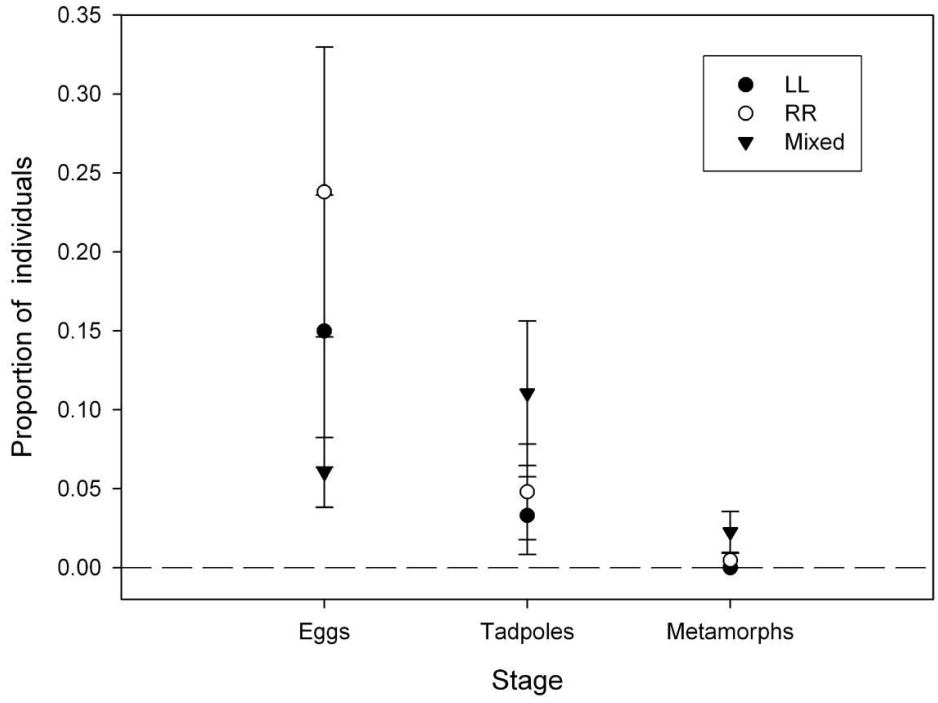


**Fig. 3:** Proportions of LL, RR and mixed genotypes at the three stages (eggs, tadpoles and metamorphs). Shown are means ± 1 SE.

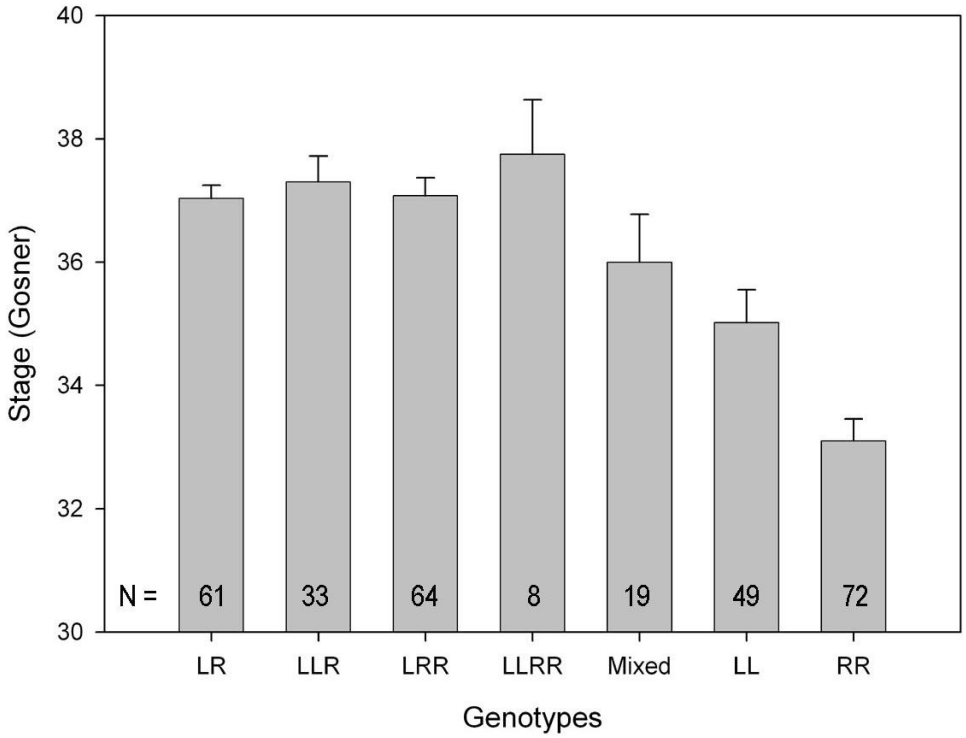


**Fig. 4:** Differences in developmental stage between genotypes for individuals that were raised from the egg stage. Larval development was measured according to Gosner (1960). Shown are means ± 1 SE.

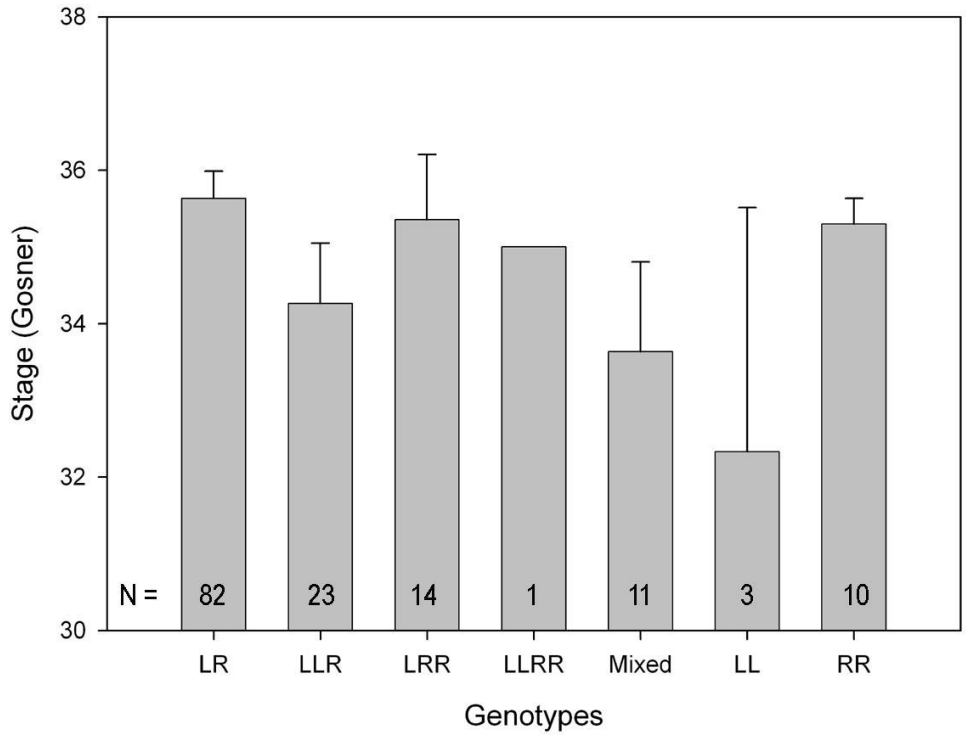


**Fig. 5:** Differences in developmental stage between genotypes for individuals from the tadpole stage in the ponds. Shown are means ± 1 SE.

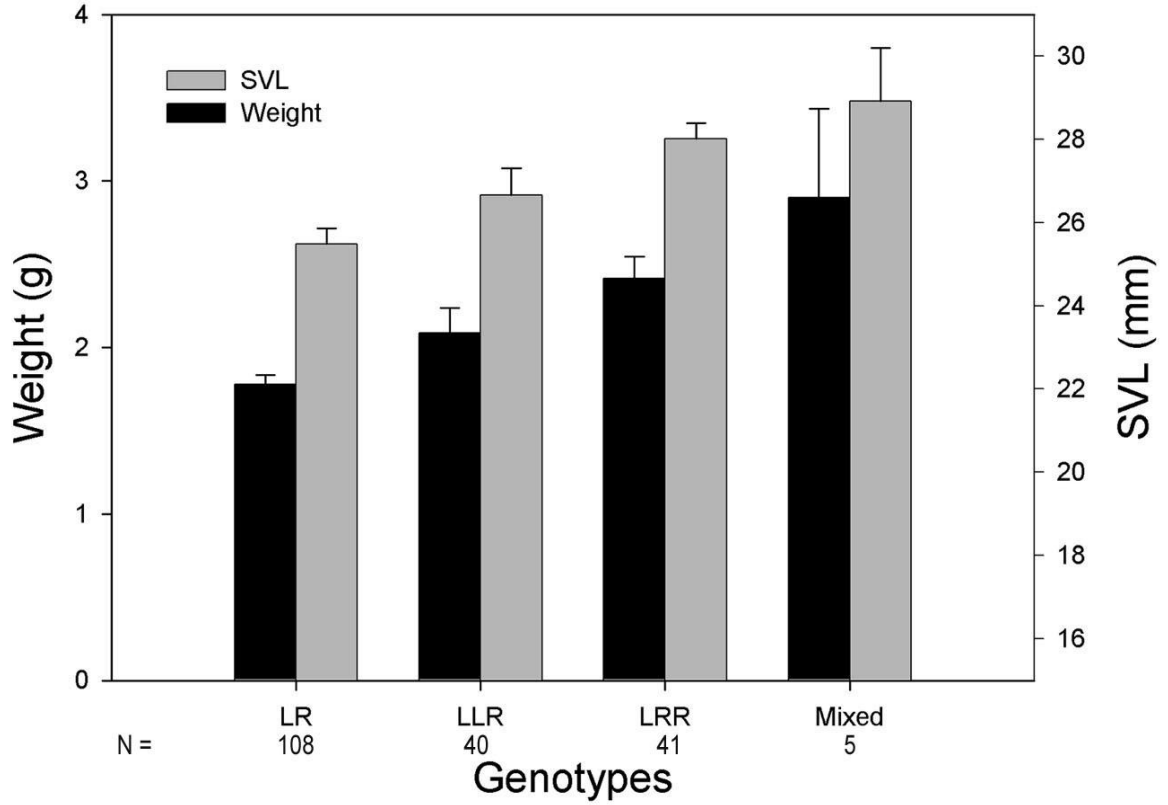


**Fig. 6:** Differences in snout-vent length (SVL) and weight between genotypes at the metamorph stage.

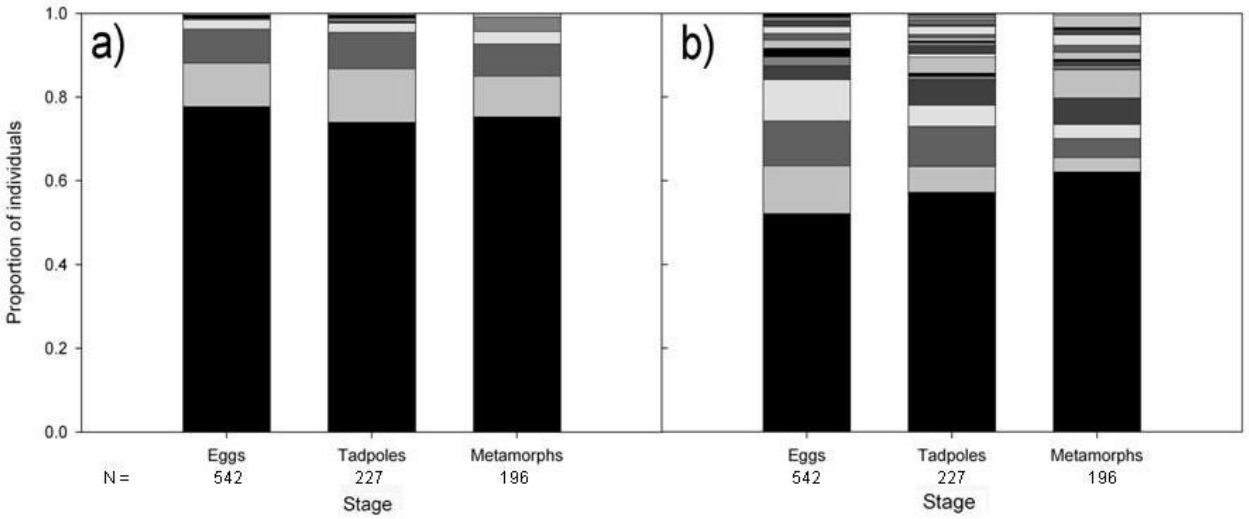


**Fig. 7:** Proportions of haplotypes for the three different stages: a) L-genome b) R-genome. Coloration refers to different haplotypes; black indicates the most common haplotype for both genomes.

## Acknowledgements

My first thanks go out to Uli Reyer for giving me the opportunity to do this thesis. I'm grateful for all his financial, scientific and administrative skills that I could rely on in all these years.

My next-to-first thanks go out to Anni Mäder for being the administrative sorceress and for never losing confidence in people (me included) and to Sandy Röthlisberger, who was moral support, walking cigarette dispenser (quit smoking, it's not good for your health), and, most important, the best lab girl imaginable. And thanks for bringing that fuzzy, lazy, drooling, adorable Fido (aka Leska) with you – the best anti-stress therapy there is!

I want to thank all the people who helped me keeping the computer chaos at the department in check, most notably Michel Nakano and Gilberto Pasinelli.

I also want to thank all the people that were part of the ecology group in these five years. I would like to thank especially Ingo Schlupp, Karin Schiegg and Gilberto Pasinelli (yet again) for providing moral support beyond scientific topics, Sonia Angelone for providing some great coffee breaks to a newcomer, Christoph Sandrock for doing the same without caffeine (quit smoking, it's not good for your health), Ditte Christiansen for sharing a MEF with me, Hansjürg Hotz, Gaston Guex, Trent Garner, Benedikt Schmidt, Christoph Vorburger and Annette Sauter for providing interactions of the (not only) scientific kind. I also thank Eline Embrechts for sharing some of her diploma thesis experience with us.

I would like to give my thanks also to Kathrin Tschannen and Katja Fink of the group of Prof. Rolf Zinkernagel at the ETH for supporting our extensive use of their FACSCalibur. Special thanks go to Eva Niederer from the IBT who always had some kind, encouraging words for us after countless hours of flow cytometry.

Jag vill tacka också våra många vänner upp i Sverige: Jon Loman, for taking care of Swedish administration and for providing excellent conditions for crossing experiments. Besides that, I am grateful for the many occasions when we were warmly welcomed for social occasions with Bodil and Torkel. I thank Jan Pröjts for providing us with many helpful details about past, present and future distributions of the Swedish herpetofauna, for taking us with him on his trip to Östergötland and for inviting us to his home. Henrik Smith and Gunilla Lindquist were always very supportive concerning our stay at Stensoffa. Many heartfelt thanks also to Bo Baldetorp and Maria Johansson at the Jubileum Institutet at the university hospital in Lund, who provided an excellent introduction into flow cytometry and were always ready to share some precious time. I want to thank the many people who shared Stensoffa with us, especially Rachel Muheim, Michi Tobler, Jean Secondi, Stefan Gödderz, Kent and Jen ☺

I thank my mother for giving me moral support all those long years and for letting me go my way, but never alone. And finally, I want to thank the universe for sending me to Martina Arioli (at her request). Because she's not only incredibly smart, funny and beautiful, but she's also the only reason for this thesis to be finished. Thank you for being patient... Jag älskar dig, lilla b!

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